ENDOCRINE AND IMMUNE PROFILES OF IMMATURE PINNIPEDS

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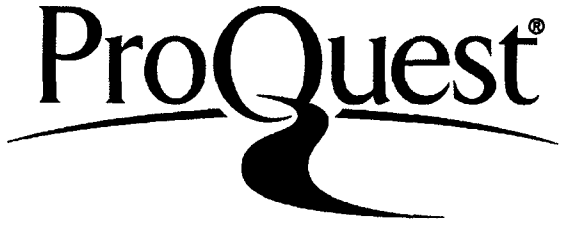

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Abstract

There is increasing interest in assessing the health of individuals and populations of pinnipeds found in the North Pacific, primarily due to population declines leading to conservation concerns. This study assessed the "health" of animals by quantifying hormones associated with fat mass (leptin), lipid and water metabolism (cortisol and aldosterone), and growth and metabolism (thyroxine and triiodothyronine) as well as circulating total and differential leukocyte counts and *in vitro* proliferation of peripheral blood mononuclear cells (PBMC). Body mass and condition are influenced by an individual's disease and nutritional state. Glucocorticoids are known to affect the immune system and may be stimulated by a multitude of factors. I hypothesized that age or body mass would influence leukocyte counts, PBMC proliferation, and hormone concentrations in Steller sea lion (*Eumetopias jubatus*) pups and that the response of cortisol to an acute stressor would impact immune parameters in juvenile harbor seals (*Phoca vitulina*). Further, given the inherent requirements of disturbance and animal handling necessary for sampling pinnipeds, the impact of these activities on endocrine and immune profiles was assessed.

Total white blood cell (WBC) counts, neutrophil counts and T cell proliferation decreased with increasing age in Steller sea lion pups. However, no relationship between body condition index and circulating concentration of hormones quantified was detected. Circulating concentrations of cortisol, thyroxine, and triiodothyronine were influenced by the rookery disturbance. However, the variation attributed to the disturbance was low and did not alter total or differential WBC counts or *in vitro* proliferation of PBMC. In harbor seals, cortisol and aldosterone concentrations increased following an acute stressor which resulted in a stress leukogram. Total WBC decreased driven primarily by the decrease in neutrophil counts with simultaneous increase in lymphocytes leading to an overall decrease in neutrophil to lymphocyte ratio. These findings highlight the endocrine system's influence on the immune system in immature pinnipeds.

Table of Contents

Page

Page

Page

List of Figures

Page

List of Tables

Page

List of Appendices

List of Abbreviations

ACTH	adrenocorticotrophic hormone
AG	axillary girth
ASLC	Alaska SeaLife Center
BCI	body condition index
BM	body mass
BrdU	bromodioxyuridine
CBC	complete blood cell
CASE	Changing Alaska Science Education
ConA	concanavalin A
CPT	cell preparation tube
DMSO	dimethyl sulfoxide
DPS	distinct population segments
ETOH	ethanol
FBS	fetal bovine serum
GC	glucocorticoid
Hb	hemoglobin
Hct	hematocrit
HPA	hypothalamic-pituitary-adrenal axis
HS	harbor seal
HE	human equivalent
ISO	isoflurane
LPS	lipopolysaccharide
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
NFS	northern fur seals
NZSL	New Zealand sea lions

OD	optical density
OC	organochlorine
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCCRC	Pollock Conservation Cooperative Research Center
Plt	platelet counts
PR	physically restrained
RIA	radioimmunoassay
RBC	red blood cell
SL	standard length
SSL	Steller sea lion
SI	stimulation index
T_4	thyroxine
T_3	triiodothyronine
WBC	white blood cell

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Chapter 1:
Introduction

1.1 Overview

There is increasing interest in assessing the health of individuals and populations of North Pacific pinnipeds primarily due to recent population declines leading to conservation concerns (Castellini *et al.*, 1993; Zenteno-Savin *et al.*, 1997; Rea *et al.*, 1998; Trumble and Castellini, 2001; Trumble and Castellini, 2002; Burek *et al.*, 2003; Burek *et al.*, 2005; Goldstein *et al.*, 2007; Atkinson *et al.*, 2008). The decline in pinniped populations has coincided with changing environmental conditions of natural and anthropogenic origin (Le Boeuf and Crocker, 2005; Guenette *et al.*, 2006; Atkinson *et al.*, 2008; Burek *et al.*, 2008). Several species of marine mammals and birds in the Northeast Pacific have been in decline since the mid-1970's (Pitcher, 1990; Loughlin, 1998; Small *et al.*, 2003; Jemison *et al.*, 2006). The number and diversity of species that experienced a decline in the Northeast Pacific led to various hypotheses based on shared ecological factors across taxa such as predation by killer whales (*Orcinus orca*), shifts in ocean productivity as a result of the Pacific Decadal Oscillation, competition with fisheries, and interspecific competition for resources.

Affected pinniped species and the focus of this dissertation include Steller sea lions (SSL; *Eumetopias jubatus*) and harbor seals (HS; *Phoca vitulina*). Population trends of HS have varied with most stocks currently being stable or increasing in numbers (Harvey *et al.*, 1990; Jemison *et al.*, 2006). However, within Alaska several populations are still reduced compared to numbers in the 1970 and1980's (Pitcher, 1990; Frost *et al.*, 1999; Hoover-Miller *et al.*, *in press*), and specifically within Prince William Sound the harbor seal population has continued to decrease (Ver Hoef and Frost, 2003). Currently, two distinct population segments (DPS) of SSL are recognized within U.S. waters: the eastern and western DPS. The western DPS is listed as endangered under the Endangered Species Act (U.S. Federal Register 62:30772–30773), having declined by more than 80% since the 1970's (Loughlin, 1998; Sease *et al.*, 2001). The cause of the population decline

and/or failure to recover of HS and SSL has been the focus of continuing research and debate (Springer *et al.*, 2003; DeMaster *et al.*, 2006; Trites *et al.*, 2007a; Atkinson *et al.*, 2008). Guenette *et al.* (2006) concluded that at different spatial and temporal scales four factors including predation by killer whales, shifts in ocean productivity as a result of the Pacific Decadal Oscillation, competition with fisheries, and interspecific competition for resources likely played a significant role in the population trends of SSL. These factors would also likely influence the population trends of other species in the same ecosystem. Excluding predation by killer whales, these factors have been proposed to lead to reduced body condition, mass or size of individuals potentially affecting the health of individuals and populations. However, there remains no clear reason for the observed decline.

Changes in body condition or mass have been associated with changes in circulating hormones in several species of pinnipeds (Jeanniard du Dot *et al.*, 2009; Ortiz *et al.*, 2003b). The neuroendocrine system influences immune responses through neuronal and hormonal signals originating from the autonomic nervous system, hypothalamic-pituitary-adrenal axis (HPA), and the renin-angiotensin-aldosterone system. Conversely, the immune system feeds back to the sympathetic/parasympathetic nervous system and the HPA axis. These finely tuned interactions are required for the survival and health of an individual and are influenced by body condition, reproductive status, and exposure to stressors (Haddad *et al.*, 2002; Weber, 2003). The mammalian immune system protects against foreign pathogens and plays a vital role in the repair of injured tissues and therefore perturbations may lead to disease. Further, seasonally breeding mammals commonly exhibit significant annual cycles in body mass and energy demands (Lincoln and Richardson, 1998; Rousseau *et al.*, 2003). These changes in body mass and/or nutritional status are characterized by alterations in concentrations of several hormones that influence body condition (Bhat *et al.*, 2003). Similar seasonality occurs in pinnipeds (Rosen and Renouf, 1997; Rosen and Renouf, 1998; Kumagai *et al.*, 2006; Mellish *et al.*, 2007; Rosen and Kumagai, 2008), which has been associated with changes in cortisol and thyroid hormones (Riviere *et al.*, 1977; Ashwell-Erickson *et al.*, 1986;

Boily, 1996; Agarwal and Marshall, 1998; Oki and Atkinson, 2004; Rosen and Kumagai, 2008).

This study assesses the "health" of individual animals by quantifying circulating concentrations of hormones associated with nutritional status and fat mass (leptin), lipid and water metabolism (cortisol and aldosterone), and growth and metabolism (thyroxine and triiodothyronine) as well as circulating total and differential leukocyte counts, and by assessing the *in vitro* proliferation of peripheral blood mononuclear cells (PBMC). Body mass and composition are influenced by an individual's disease and nutritional state and may act as a proxy for assessing an individual's or population's health. Therefore, age and body mass (or condition) is proposed to be an important factor in the health of juvenile HS and SSL pups. I hypothesize that age or body size will be associated with: 1) circulating leukocyte counts; 2) peripheral blood mononuclear cell proliferation; 3) baseline hormone concentrations; and 4) changes in hormone concentrations and immune parameters in response to exogenous adrenocorticotrophic hormone (ACTH) stimulation.

1.1.1 Objectives

Objective 1: Evaluate the influence of age on the total and differential leukocyte counts and *in vitro* proliferation of PBMC in SSL pups during the early post-natal period.

Objective 2: Evaluate the influence of body condition on circulating concentrations of hormones associated with nutritional status and fat mass (leptin), lipid and water metabolism (cortisol and aldosterone), and growth and metabolism (thyroxine and triiodothyronine) during the early postnatal period in SSL pups.

Objective 3: Determine the impact of rookery disturbance and animal handling necessary for sampling wild pinnipeds on endocrine (cortisol, aldosterone, thyroxine, triiodothyronine, and leptin) and immune (leukocyte counts and *in vitro* proliferation of PBMC) profiles of SSL pups.

Objective 4: Evaluate the physiological response to an acute stressor (exogenous ACTH) on endocrine (cortisol, aldosterone, thyroxine, and triiodothyronine

concentrations) and immune parameters (total and differential leukocyte count and *in vitro* proliferation of PBMC) in juvenile HS.

1.2 Life History of Pinnipeds

Pinnipeds are a monophyletic group having diverged from a shared arctoid carnivore ancestor that reentered the sea approximately 25 million years ago (Berta and Adam, 2001) and subsequently diverged into three families: Otariidae (sea lions and fur seals), Odobenidae (one extant species, the walrus), and Phocidae (true seals). All extant pinnipeds are amphibious, dividing their time between foraging at sea and hauling out on land or sea ice to reproduce, molt, and rest. There is striking diversity in life history traits (e.g. mating and lactation strategies, body size, degree of sexual dimorphism) between and within the three pinniped families, likely driven by multiple factors including thermoregulation, distribution of resources, predation pressures, breeding substrate and phylogenetic constraints (Costa, 1991; Boness and Bowen, 1996; Boyd, 1998).

In general pinnipeds are aggregate, seasonal breeders with mating occurring in water or on land (or ice) resulting in highly synchronized terrestrial parturition of a single pup. Gestational periods are relatively long ranging between 9.5 and 16 months of which 1.5-5 months is an embryonic diapause (Bowen, 1991). Adult female phocids are generally larger than otariids resulting in phocid pups having larger absolute mass at birth and faster postnatal growth rates compared to otariid pups (Bowen, 1991; Costa, 1991; Kovacs and Lavigne, 1992). When compared on a mass-specific basis of the adult female, mass of pups at birth are similar between phocids and otariids (Kovacs and Lavigne, 1986; Kovacs and Lavigne, 1992) while otariid pups are larger (and older) at weaning than phocids (Bowen, 1991; Costa, 1991). As with all placental mammals, supporting the nutritional needs of an offspring through weaning is the period of the highest energetic cost of reproduction (Millar, 1977).

Though overly simplistic, several generalizations are often made about the life history strategies of phocids and otariids. Shorter lactation periods ranging between 4-50 days (Bowen, 1991) are found in phocids and are characterized by fat content in the milk

between 40-60% (Schulz and Bowen, 2004) and a larger energy store in the form of a subcutaneous blubber layer in the adult female phocid (per kilogram of body mass) compared to otariid (Costa, 1991). This large blubber layer supports both milk production and the nutritional requirements of the adult female during the fasting period associated with nursing. The pups are abruptly weaned when the adult female leaves the pup in order to forage, though some smaller phocids such as the harbor seal forage while supporting the nutritional needs of their pups (Boness *et al.*, 1994). The large blubber layer of the pup, which is acquired during the relatively short nursing period, provides the nutritional support for the pup during its post weaning fast and while it develops swimming and foraging skills (Bowen, 1991; Mellish *et al.*, 1999; Frost *et al.*, 2006; Clark *et al.*, 2007).

The relatively longer lactation period for otariids of 17-77 weeks are characterized by fat content in milk ranging between 14-44% (Arnould *et al.*, 1996; Boness and Bowen, 1996; Arnould and Hindell, 1999) and smaller subcutaneous blubber layer in the adult female. Otariids have "foraging cycle strategy" of alternating forage bouts between nursing periods in order to meet their own nutritional needs as well as those of the pup. Otariid pups remain at the rookery, fasting until the adult female returns from foraging trips (Bonner, 1984; Oftedal *et al.*, 1987; Trillmich, 1990; Costa, 1991). Foraging trips will last hours to days and increase in duration as the pup matures (Maniscalco *et al.*, 2006). The pup will begin to forage with the adult female to supplement its nutritional needs and is gradually weaned, generally prior to the birth of the new pup.

The current study focuses on two species: one otariid, the SSL and one phocid, the Pacific HS. Therefore, the remainder of this section will focus on the specific life history strategies and conservation status of these two species.

1.2.1 Steller Sea Lions

The SSL is the largest otariid and exhibits dramatic sexual dimorphism (male's annual average body mass 556 kg, breeding season 1,120 kg; females' annual average

body mass 263 kg, breeding season 350 kg). Similar to other otariids, SSL have seasonal and highly synchronized parturition and mating, which requires a return to land. This reproductive strategy of terrestrial parturition is characterized by large aggregations of territorial bulls, adult females, yearlings and pups present seasonally on the rookery.

At the time of birth, pups remain with the adult female during the perinatal period, ranging between 1.7 to 17.1 days (Merrick *et al.*, 1987; Hood and Ono, 1997; Maniscalco *et al.*, 2006). Pups have continuous access to the female for nursing during the perinatal period, which ends when the female returns to the sea for the first foraging trip since giving birth, leaving the pup unattended on land for several hours to days. For the remainder of the lactation period the female will alternate between short foraging trips to sea and visits to land to nurse the pup. The lactation period lasts about one year during which the female may be pregnant with next year's pup.

SSL are distributed along the North Pacific Rim from northern Japan to California (Loughlin *et al.*, 1984). Within U.S. waters there are currently 2 federally recognized distinct population segments (DPS): the eastern and western DPS, divided along the 144° W longitude (Cape Suckling, Alaska), based upon variation in mitochondrial control-regions (Bickham *et al.*, 1996; Bickham *et al.*, 1998). The eastern stock includes rookeries in California, Oregon, British Columbia, and southeastern Alaska while the western stock is comprised of rookeries within Prince William Sound, the Bering Sea, Central and Western Gulf of Alaska, the Aleutian Islands, Russia, and the Kuril Islands. Within U.S. waters, both DPS fall under the jurisdiction of the NOAA Fisheries with the western DPS currently listed as endangered and the eastern DPS as threatened under the Endangered Species Act and depleted throughout its ranged under the Marine Mammal Protection Act.

The SSL population has declined by more than 80% since the 1970's (Sease *et al.*, 2001). There are several potential factors that may have lead to the decline and/or lack of recovery including predation, disease, contaminants, and nutritional stress (Atkinson *et al.*, 2008). While the mechanistic cause of the decline is a matter of continuing debate (Springer *et al.*, 2003; Fritz and Hinckley, 2005; DeMaster *et al.*, 2006; Trites *et al.*,

2007b; Atkinson *et al.*, 2008), several studies have suggested a reduction in juvenile survival (York, 1994; Holmes and York, 2003) and a long-term decrease in natality rates (Holmes and York, 2003; Holmes *et al.*, 2007). In spite of the dramatic population decline, Baker *et al.*, (2005) found the diversity of haplotypes and nucleotides within the mitochondrial control region were high and moderate, respectively, across the entire range of SSL, concluding that there was no evidence of a genetic bottleneck.

1.2.2 Harbor Seals

HS are currently classified into four recognized subspecies found in either the Atlantic (North America: *Phoca vitulina concolor;* Europe: *Phoca vitulina vitulina*) or Pacific Oceans (North America: *Phoca vitulina richardsii*; Asia: *Phoca vitulina stejnegeri*). A fifth subspecies, based upon morphology and behavior has been proposed for a freshwater population found in Quebec, Canada (*Phoca vitulina mellonae*) (Smith and Lavigne, 1994). The classification of two subspecies in the Pacific HS was originally based on morphological differences including body size, coat color and cranial morphology (Temte *et al.*, 1991). However, more recent molecular studies have not supported the division of the Pacific HS into two subspecies (Westlake and O'Corry-Crowe, 2002).

Phoca vitulina concolor and *Phoca vitulina richardsii* can be found coastally within U.S. waters of the Atlantic and Pacific Oceans, respectively. For management purposes NMFS currently divides the two subspecies into seven stocks including one Atlantic (Western North Atlantic) and six Pacific (Bering Sea, California, Gulf of Alaska, Oregon-Washington coastal, Southeast Alaska, and Washington Inland). Population trends have varied for the different management stocks with most stocks stable or increasing since the Marine Mammal Protection Act was enacted in 1972 (Harvey *et al.*, 1990; Jemison *et al.*, 2006). However, the Gulf of Alaska stock may be continuing to decline and is still reduced compared to population numbers in the 1970 and1980's (Pitcher, 1990; Frost *et al.*, 1999; Hoover-Miller *et al.*, *in press*) and specifically in Prince William Sound (Ver Hoef and Frost, 2003) while other populations in southeastern

Alaska, excluding Glacier Bay which has declined (Mathews and Pendleton, 2006), have increased (Small *et al.*, 2003).

HS are small phocids displaying some sexual dimorphism with adult male (average body mass 115 kg; average length 175 cm) being larger than female harbor seals (80 kg; 160 cm). They are polygamous, aquatic breeders with males showing plasticity in mating behaviors, which often involves occupying underwater territories where they perform vocal displays for females (Bowen *et al.*, 2006). Females have been observed to first give birth between 4 and 6 years of age and continue to grow after sexual maturity until about 10 years (Markussen *et al.*, 1989). This has lead to numerous studies to assess the effect of maternal body condition and age on birth and weaning mass and the survival of the pup (Ellis *et al.*, 2000; Bowen *et al.*, 2001).

HS give birth to a single pup, which actively swims and dives in shallow waters within hours of birth and throughout the nursing period. Similar to other phocids, pups have a short nursing period lasting 24 days (Bowen *et al.*, 1992; Bowen *et al.*, 2001) resulting in a rapid postpartum growth associated with a large deposit of lipid reserves in the blubber layer. This foraging and lactation cycle differs somewhat from other phocids that fast during the entire nursing period (ranging 4-50 days). The foraging and nursing cycle observed in HS is similar to, though comparatively shorter than, those observed in otariids (Boness *et al.*, 1994; Bowen *et al.*, 2001). Bowen *et al.*, (1992) suggest the harbor seal lactation strategy is the result of smaller lipid reserves in the adult female compared to other phocids.

Given the high energy demand and rapid growth and development in both harbor seal and Steller sea lion juveniles and pups, I hypothesize that age or body size will be associated with circulating concentrations of hormones, leukocyte counts and *in vitro* proliferation of PBMC.

1.3 Body Condition

Disease and nutrition are considered the major external biotic factors influencing growth in vertebrates (Vander *et al.*, 1986). Body condition indices (BCI) have been

applied in livestock to assess health and production measures, e.g. sheep (Caldeira *et al.*, 2007), and to wild populations of terrestrial mammals, e.g. bears (Cattet *et al.*, 2002). Increasingly there has been an interest in quantifying body condition in pinnipeds as a measure of health and fitness (Castellini and Calkins, 1993; Arnould and Hindell, 1999; Jonker and Trites, 2000; Pitcher *et al.*, 2000; Trites and Jonker, 2000; Arnould and Warneke, 2002; Rea, 2002). BCI in pinnipeds have been proposed based on various combinations of mass, standard length, axillary girth, and blubber thickness (Pitcher *et al.*, 2000; Trites and Jonker, 2000; Rea, 2002). These calculated indices have been used for prediction of energy reserves, reproductive success and pup survival.

Body mass and condition are influenced by an individual's disease and nutritional state and therefore may act as a proxy for assessing an individual's or population's health. The potential impact of body condition on an animal's ability to survive would be greatest during times of high energy demands such as pregnancy, lactation and periods of development and rapid growth. Adult female otariids (Antarctic fur seals, *Arctocephalus gazella*) exhibited reduced pup production in years following an El Niño Southern Oscillation (Guinet *et al.*, 1994) and larger, older females gave birth to larger pups (Lunn and Boyd, 1993). In southern elephant seals (*Mirounga leonina*) larger females gave birth to larger pups (regardless of pup sex) and male pups were heavier than female pups at the time of birth (Arnbom *et al.*, 1994). Similarly, mass of HS at birth and postnatal growth rates were influenced by the mass of the adult female, with heavier females giving birth to heavier pups with a faster growth rate (Ellis *et al.*, 2000; Bowen *et al.*, 2001). Body mass and lipid stores in HS were correlated with survival during the first year of independence (Muelbert *et al.*, 2003; Harding *et al.*, 2005). Overall, there is strong evidence supporting the importance of body mass at birth or weaning as predictive of survivorship of pups in phocids and otariids (Baker and Fowler, 1992; Kjellqwist *et al.*, 1995; Harding *et al.*, 2005). However, the relationships between BCI or mass with health parameters such as complete blood cell (CBC) count, immune function (e.g. PBMC proliferation), or circulating hormone concentrations have not been fully explored in pinnipeds.

Seasonally breeding mammals such as pinnipeds commonly exhibit robust annual cycles in body mass and energy demands (Lincoln and Richardson, 1998; Rousseau *et al.*, 2003). BCI or body mass and nutritional status showed seasonality in phocids (Ryg *et al.*, 1990; Beck *et al.*, 1993; Renouf *et al.*, 1993; Nilssen *et al.*, 2001; Chabot and Stenson, 2002) and otariids (Kumagai *et al.*, 2006; Rea *et al.*, 2007; Williams *et al.*, 2007; Jeanniard du Dot *et al.*, 2008). Changes in body mass and/or nutritional status are characterized by alterations in concentrations of many hormones that are related to body condition (Bhat *et al.*, 2003). Similar seasonality occurs in pinnipeds (Rosen and Renouf, 1997; Rosen and Renouf, 1998; Kumagai *et al.*, 2006; Mellish *et al.*, 2007; Rosen and Kumagai, 2008), which has been associated with changes in cortisol and thyroid hormones (Riviere *et al.*, 1977; Ashwell-Erickson *et al.*, 1986; Boily, 1996; Oki and Atkinson, 2004; Rosen and Kumagai, 2008; Jeanniard du Dot *et al.*, 2009). Specifically, decreases in lipid stores and mass were associated with an increase in circulating cortisol concentration and decrease in total T_4 and T_3 concentrations in SSL during experimental food restriction (Rosen and Kumagai, 2008; Jeanniard du Dot *et al.*, 2009). Many of these studies have focused on periods of high energy demands such as lactation, natural or experimental fasting, or molting. Previous studies assessing the relationship of body mass or condition with hormones in SSL primarily focused on juvenile and sub-adults (Mellish *et al.*, 2006; Jeanniard du Dot *et al.*, 2008; Rosen and Kumagai, 2008; Jeanniard du Dot *et al.*, 2009; Rea *et al.*, 2009) and did not assess the impact of mass on immune parameters. Conversely, studies on HS have generally focused on pups and the influence of body mass or age on either changes in hormone concentrations associated with weaning (Riviere *et al.*, 1977; Ashwell-Erickson *et al.*, 1986; Boily, 1996) or the postnatal development of the immune system by assessing leukocyte counts and *in vitro* proliferation of PBMC (Swart *et al.*, 1993; Ross *et al.*, 1994; Nielsen, 1995; Levin *et al.*, 2005; Kakuschke *et al.*, 2008a). Therefore, this study assesses how age or body size are associated with circulating concentrations of hormones as well as circulating total and differential leukocyte counts and the *in vitro* proliferation of PBMC in SSL pups. Further, this study seeks to build upon the abundance of research on hormone concentrations,

leukocyte counts and PBMC proliferation in HS by assessing how body mass influences the physiological response to an acute stressor (exogenous ACTH) on cortisol, aldosterone, thyroxine and triiodothyronine concentrations, total and differential leukocyte counts and *in vitro* PBMC proliferation in juvenile HS.

1.4 Endocrine System

1.4.1 Hypothalamic-Pituitary-Adrenal Axis

Hypothalamic-pituitary-adrenal (HPA) axis activity varies between and within species depending on life history traits, environmental conditions, breeding status, sex and age. The HPA axis and its endpoint products, cortisol, the prominent glucocorticoid (GC) hormone in pinnipeds (DeRoos and Bern, 1961; Sangalang and Freeman, 1976) and aldosterone, a mineralocorticoid, play key roles in physiology and behavior of animals. GC are synthesized in the zona fasciculata of the adrenal cortex following stimulation by ACTH and inhibit peripheral tissues such as skeletal muscle from utilizing glucose and enhance the mobilization of fat stores to non-neuronal tissues. In pinnipeds, cortisol plays a significant role during fasting and/or lactation (Engelhard *et al.*, 2002; Guinet *et al.*, 2004; Jeanniard du Dot *et al.*, 2009), molt (Riviere *et al.*, 1977; Ashwell-Erickson *et al.*, 1986), and potentially during diving (Zapol *et al.*, 1979).

Pinnipeds exhibit a typically mammalian structure in the adrenal gland with an increase in connective tissue and surface lobulation (Ridgway, 1972). Adrenal gland mass increased with age in HS with relative adrenal gland mass decreasing with body mass (Sucheston and Cannon, 1980; Bragulla *et al.*, 2004). The medullary area to cortex ratio has been described as low (Sucheston and Cannon, 1980). The cortex shows typical mammalian zonation with the of zona glomerulosa accounting for nearly half the cortical area, which is larger compared to terrestrial mammals (DeRoos and Bern, 1961; Sucheston and Cannon, 1980). The increase in area of the zona glomerulosa was attributed to the need to maintain electrolyte balance while living in a marine environment. Aldosterone is secreted from the cells of the zona glomerulosa and is regulated by the renin-angiotensin system in pinnipeds (Malvin *et al.*, 1975; Ortiz *et al.*,

2000; Houser *et al.*, 2001; Ortiz *et al.*, 2003a; Ortiz *et al.*, 2006). Secretion of aldosterone is stimulated by angiotensin II and induces the absorption of Na^+ in the distal tubule of the nephron maintaining water and electrolyte balance. Previous studies have also demonstrated that aldosterone is secreted in response to a stressor in mammals. For example, aldosterone concentrations increased during acute stress response (Thomson and Geraci, 1986; St. Aubin *et al.*, 1996; Gardiner and Hall, 1997; Contreras *et al.*, 2004; Romano *et al.*, 2004) and during exogenous ACTH challenges (St. Aubin and Geraci, 1986; Gulland *et al.*, 1999) in association with increased concentrations of cortisol, a well known stress hormone.

Stress has been defined as a state of threatened homeostasis resulting from internal or external environmental change (Stratakis and Chrousos, 1995; Leonard, 2005). Stress falls into one of two categories based on the duration or frequency of the stressor. Stress marked by a long duration or with frequent recurrence over an extended period is classified as chronic, while stressors having a sudden onset, sharp rise, and short course are classified as acute. Animals or populations in poor body condition may be experiencing chronic stress associated with a reduction in prey abundance or quality often referred to as nutritional stress (Merrick *et al.*, 1987; Calkins *et al.*, 1998; Anderson *et al.*, 1999; Trites and Donnelly, 2003). Capture and handling methods routinely used during blood collection in wild mammal populations is classified as an acute stressor and has been shown to result in elevated cortisol concentrations (Engelhard *et al.*, 2002; Romero *et al.*, 2008) and alter hematological parameters (Castellini *et al.*, 1996; Cattet *et al.*, 2003). Cortisol concentration (approximately 130 ng/ml) was elevated following capture in juvenile SSL compared to concentrations measured following acclimation to temporary captivity (74 ± 22 ng/ml) (Petrauskas *et al.*, 2008) and from captive juvenile and adult SSL (Mashburn and Atkinson, 2004; Rosen and Kumagai, 2008).

Elevated cortisol concentrations have been shown to suppress immune function including PBMC proliferation in piglets (Westly and Kelley, 1984) while having no influence in lambs (Minton and Blecha, 1990; Coppinger *et al.*, 1991) or calves (Manak, 1986). The effect of elevated GC on immune parameters is dependent on several

variables including the concentration of GC induced by an acute stressor, the immune parameter or function, and the species being studied (Anderson *et al.*, 1999; Bilandzic *et al.*, 2006). An acute stress response is generally considered adaptive while exposure to chronic stressors and the associated prolonged elevated concentrations of GC is detrimental to the health and survival of individuals. Morphological changes in adrenal gland size, medulla to cortex ratio, and individual zones have been associated with chronic stress and disease. Adrenal necrosis associated with herpes virus inclusions in harbor seal pups that died during rehabilitation showed marked lymphoid atrophy in the thymus, spleen and lymph nodes and significant lymphopenia (Gulland *et al.*, 1997; Gulland *et al.*, 1999), though the mortality was not attributed to adrenal insufficiency. The acute lymphoid depletion observed in several lymph nodes (tracheobronchial, axillary, mesenteric or jejuna) in leopard seals (*Hydrurga leptonyx*) was attributed to endogenous steroid release (Gray *et al.*, 2006). However, previous studies on the acute stress response in pinnipeds (St. Aubin and Geraci, 1986; Gulland *et al.*, 1999; Mashburn and Atkinson, 2008) have not assessed the impact of an acute stressor on the total and differential leukocyte count or *in vitro* proliferation of PBMC. Therefore, this study assessed the effect of exogenous ACTH on *in vitro* PBMC proliferation in addition to total and differential leukocyte counts in HS as a means of assessing the impact of an acute stressor.

1.4.2 Thyroid Gland and Hormones

Thyroid hormones including total thyroxine (T_4), free T_4 and total triiodothyronine (T_3) are essential for growth and development, metabolism and thermoregulation. Thyroid gland mass increased with age with sexually mature adult thyroid weighing about twice that of immature harbor seals (Harrison *et al.*, 1962); however, no difference in mass was observed between sexes in pups or adults (Harrison *et al.*, 1962; Little, 1991). Two periods of growth in the thyroid gland were suggested, the first period occurred during late gestation with the second occurring near sexual maturation coinciding with an increase in body mass. Thyroid hormones were also

affected by age in seals with pups having higher concentrations in T_4 and T_3, which has been attributed to the high metabolic and thermoregulatory needs of pups (Hall *et al.*, 1998; Haulena *et al.*, 1998; Woldstad and Jenssen, 1999).

1.4.3 Leptin

Leptin is secreted by adipose tissue (Friedman and Halaas, 1998; Kershaw and Flier, 2004) and circulating concentrations have been correlated with fat stores and respond to changes in energy balance in terrestrial mammals (Friedman and Halaas, 1998). Specifically, in human and rodents there is a strong positive relationship between leptin concentrations and body fat mass (Ahima *et al.*, 1996). In horses (Buff *et al.*, 2002), cows and sheep (Chilliard *et al.*, 2001) body condition and fat mass were similarly related to serum leptin concentrations. Further, there is increasing evidence that leptin has systemic effects including regulation of neuroendocrine, immune function and development. Reduced circulating concentrations of leptin during malnutrition have been shown to directly influence acquired immunity by suppressing T lymphocyte proliferation (Lord *et al.*, 1998).

Leptin concentration has a clear relationship with fat mass in some mammals; however, the relationship has not been observed in other mammalian species. These findings have lead some researchers to suggest that carnivores lack a detectable relationship between body condition and leptin concentrations (Ahima and Flier, 2000; Nieminen *et al.*, 2001; Arnould *et al.*, 2002). For example, in terrestrial mammals, food restrictions leading to a loss in body fat mass was not associated with changes in leptin concentration in the raccoon dog (*Nyctereutes procyonoides*) (Nieminen *et al.*, 2004). Similarly, leptin concentrations in northern elephant seal (*Mirounga angustirostris*) (Ortiz *et al.*, 2001; Ortiz *et al.*, 2003b) and Antarctic fur seal pups (Arnould *et al.*, 2002) did not correlate to fat or body mass. However, adult elephant seals underwent a significant decrease in leptin associated with decreasing lipid stores while fasting during periods of reproduction and molt (Guilherme *et al.*, 2004). During periods of fasting, serum leptin concentrations were higher in adult elephant seals (Guilherme *et al.*, 2004)

compared to pups (Ortiz *et al.*, 2001; Ortiz *et al.*, 2003b). These findings may be reflective of the role of the blubber layer during fasting and postnatal development with the lower levels of leptin observed in otariid pups being reflective of the comparatively low lipid stores (Brandon *et al.*, 2005) and smaller blubber layer (Castellini *et al.*, 1993; Trites and Jonker, 2000) observed in otariid pups.

Fluctuating leptin concentrations not associated with changes in fat or body mass have been previously reported in pinnipeds (Arnould *et al.*, 2002; Mashburn and Atkinson, 2008). Mashburn and Atkinson (2008) reported a large amount of variability in serum leptin concentrations in response to an exogenous ACTH challenge in juvenile SSL. Difficulty in quantifying leptin in SSL (Rosen and Kumagai, 2008) and other pinnipeds (Arnould *et al.*, 2002; Ortiz *et al.*, 2003b) have been reported with low concentrations or no correlation to body mass or lipid stores, leading several researchers to propose that leptin is not a prominent signal of fat reserves in pinnipeds (Ortiz *et al.*, 2001; Arnould *et al.*, 2002; Rosen and Kumagai, 2008). Therefore, the relationship of leptin and body condition or mass remains unclear in pinnipeds. This study assessed leptin concentrations in SSL pups of known age and mass during the postnatal period to assess the relationship between mass and leptin concentrations during a period of rapid growth associated with increasing lipid stores in otariids.

1.5 Immune System

1.5.1 Immune Organs and Leukocytes

The vertebrate immune system has two types of responses, the innate (natural) and acquired (adaptive) responses. The innate response is the host defense that exists prior to exposure to a specific antigen and involves phagocytic cells (neutrophils, monocytes, and macrophages), inflammatory mediators (basophils and eosinophils) and natural killer cells. Neutrophils circulate in the peripheral blood for 7-10 hrs (e.g. human) following which they migrate into tissues where they will remain for a few days (Goldsby *et al.*, 2003). Monocytes circulate in the blood for about 8 hrs (e.g. human) and then migrate into tissues and differentiate into macrophages or dendritic cells (Goldsby *et al.*,

2003). During an infection, the number of circulating neutrophils increases (leukocytosis) and are generally the first cells to arrive at a site of inflammation. Similarly, circulating neutrophil counts increase rapidly with a concurrent decrease in lymphocyte and monocyte counts during an acute stress response in association with elevated GC concentrations (Dhabhar *et al.*, 1995; Dhabhar *et al.*, 1996; Anderson *et al.*, 1999).

Lymphocytes are responsible for the adaptive immune response in vertebrates with the majority of lymphocytes found in lymph. Lymphocytes mature and become committed to a particular antigenic lineage in the thymus (T cells) or in the bone marrow (B cells). Mature lymphocytes leave the primary lymphoid organs and circulate in the blood and lymphatic system. The adaptive response is generated in the spleen, lymph nodes, and the mucosa-associated lymphoid tissue leading to the proliferation of antigen-specific B and T lymphocytes.

The primary organs of the mammalian immune system are the thymus and bone marrow where lymphocytes mature. The thymus in pinnipeds has not been well described in the literature. Smodlaka *et al.*, (2009) noted that the thymus of one juvenile ringed seal (*Pusa hispida*) was bilobed and easily identified while only remnants of the thymus were present in an adult. Involution of the thymus associated with aging has been described in several species including the dog (Newman, 1971), dolphin (Clark *et al.*, 2005), cattle (Lubis *et al.*, 1982) and human (Suster and Rosai, 1990). It is therefore likely that the thymus in pinnipeds undergoes involution following the onset of sexual maturity as in terrestrial mammals.

The secondary lymphoid organs include skin, lymph nodes, and the spleen. Though often overlooked, the first barrier to infection is the skin and the mucosal membranes of an animal. Histology of skin in several species of pinnipeds showed increased thickness of the epidermis and numerous sebaceous glands (Montagna and Harrison, 1957). The integument of several pinniped species, including the harbor seal were positive for the presence of β-glucan receptors in association with skin glands, outer epithelial sheath of the hair follicles and in the epidermis, supporting a role of pinniped skin in the innate immune system (Meyer *et al.*, 2008).

The spleen of pinnipeds appears to have several functions including immunologic, haematopoiesis and blood storage (Schumacher and Welsch, 1987). The spleen has been described as moderately large (Slijper, 1958) and increases in mass with body mass (Scheffer, 1960) but accounts for approximately 0.11-0.40 % of the total body weight in Cape fur seal (Otariid; *Arctocephalus pusillus*) (Stewardson *et al.*, 1999) and the northern fur seal (Otariid; *Callorhinus ursinus*) (Scheffer, 1960), which is comparable to terrestrial mammals. The spleen is enclosed in a thick capsule with prominent trabecule and innervations of smooth muscle cells (Schumacher and Welsch, 1987; Gray *et al.*, 2006). Stewardson *et al.*, (1999) determined that the spleen, along with the heart and liver, in the Cape fur seal grew at a faster rate than the body and other organs. The spleen grew approximately 33% per year until at least 10 years of age with varying degrees of development in the red and white pulp of the parenchyma, which may indicate ontogeny changes, disease status, or both. The noted size and growth of the spleen has generally been attributed to the spleen's role in sequestering red blood cells and diving ability of these species (Cabanac *et al.*, 1989; Ponganis *et al.*, 1997). However, the spleen in pinnipeds (both phocids and otariid) has a prominent lymphatic part with well developed follicles and numerous plasma cells in the white pulp and eosinophils in the red pulp. The presence of eosinophils in the spleen was attributed to the relatively high load of gastrointestinal parasites (Schumacher and Welsch, 1987; Stewardson *et al.*, 1999; Gray *et al.*, 2006). Eosinophils are motile phagocytic cells that can migrate from the blood into the tissue spaces and are thought to play a role in the defense against parasitic organisms.

The lymph nodes of pinnipeds exhibit the general mammalian pattern both structurally and histologically with lymphoid follicles located within the cortex (Welsch *et al.*, 1997; Gray *et al.*, 2006). In both phocids and otariids, the capsule and trabecular penetrating the parenchyma of lymph nodes are broad containing smooth muscle cells within a network of collagenous and elastic fibers. Similar structures have been found in odontoceti (toothed whales) and have been proposed to enable active movement and filtration of lymph fluid by capsular contraction (Romano *et al.*, 1993; Cowan and Smith, 1999). The degree of development of lymphoid tissue was variable between individuals

(Stewardson *et al.*, 1999). Kutyrev *et al.*, (2008) found ontogenetic changes in the mesenteric lymph nodes of the Baikal Seal (*Pusa sibirica*) including a dramatic increase in the capsule and trabeculae with age. These changes were associated with a decrease in the relative area of the cortex, secondary lymph follicles, paracortical zone and medulla resulting in an overall decrease of the corticomedullary index with age.

The mesenteric lymph nodes of the harbor and grey (*Halichoerus grypus*) seals exhibited numerous fully developed large secondary follicles and broad T cell regions with most follicles appearing as germinal centers indicative of an immunological response to an antigenic challenge (Welsch *et al.*, 1997). These mesenteric lymph nodes were collected from healthy individuals lacking any gross signs of disease and these observations were attributed to the exposure to antigens within the coastal marine or terrestrial environment.

Lymph nodes obtained during epidemics of harbor seal (Schumacher *et al.*, 1990) or from compromised leopard seals (Gray *et al.*, 2006) were lymphocyte depleted compared to other mammals or healthy animals of the same species (Stewardson *et al.*, 1999). The observed lymphocyte depletion was attributed to either the natural disease process or the result of endogenous steroid release associated with restraint and sedation (Gray *et al.*, 2006). Similar to findings in the spleen, eosinophils were noted in the lymph nodes in both phocids (Boily, 1996) and otariids (Simpson and Gardner, 1972; Welsch *et al.*, 1997) and were attributed to the prevalence of parasites in these species.

1.5.2 Hematology and PBMC Proliferation

Hematology has been routinely used to assess the health of individuals and populations of pinnipeds including SSL (Castellini *et al.*, 1993; Bishop and Morado, 1995; Rea *et al.*, 1998) and HS (McConnell and Vaughan, 1983; Ross *et al.*, 1993; Morgan *et al.*, 1998; Lander *et al.*, 2003; Hasselmeier *et al.*, 2008). Further, some hematological parameters such as hematocrit (Hct), hemoglobin (Hb), white blood cell (WBC) and platelet count (Plt) have been used for assessing body condition and nutritional status or stress in several species of pinnipeds (Thompson *et al.*, 1997; Rosen

et al., 2004; Trumble *et al.*, 2006). Across mammalian species and in both sexes the number of circulating total WBC count increased with body mass in adults (Nunn *et al.*, 2003; Nunn *et al.*, 2009). This increase was driven predominantly by circulating neutrophil and monocyte counts with a concurrent decrease in lymphocyte counts. Significant differences in total and differential WBC counts were found between immature and mature mammals across taxa including carnivores (Nunn *et al.*, 2009). The strongest difference was found in circulating lymphocyte counts with immature individuals having higher lymphocyte counts than both mature males and females of the same species. Similar observations have been reported in pinnipeds. Nielsen (1995) found neutrophils (%) increased while lymphocytes (%) decreased with age (1.5-17.5 yrs) in HS. The decrease in lymphocytes (%) was associated with a decrease in response to *in vitro* mitogen stimulation of PBMC. However, Kakuschke *et al.*, (2008b) reported higher PBMC proliferation in HS pups compared to adults. Taken together these findings are suggestive that the age-related changes in PBMC proliferation are not linear with age but that pups undergo an initial increase prior to a decrease in proliferation of PBMC in adults. Similar studies have not been done on SSL. Mellish *et al*,. (2006) sampled SSL pups (6–10 months) and juveniles (1–3 years) concluding that pups had significantly lower WBC counts than juveniles; however, differential leukocyte counts and PBMC proliferation were not included in the study. No study has assessed the age-related change in total and differential leukocyte counts or PBMC proliferation in SSL less than 6 months of age. Therefore, this project sampled SSL pups during the early postnatal period to assess changes in total and differential leukocyte counts and proliferation of PBMC as stimulated by concanavalin A (ConA) and lipopolysaccharide (LPS).

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Chapter 2:

Steller sea lion (*Eumetopias jubatus*) pups undergo a decrease in circulating white blood cells and the ability of T cells to proliferate during early post-natal development[1]

2.1 Abstract

Postnatal changes in circulating immune components and peripheral blood mononuclear cells (PBMC) proliferation were assessed in Steller sea lion (SSL; *Eumetopias jubatus*) pups. Blood samples were collected for complete blood cell counts including total and differential white blood cell (WBC) counts from 46 pups ranging in age from 5 to 38 days old. Total WBC and neutrophil counts decreased in association with increased age of the pups. The ability of PBMC to proliferate was assessed by *in vitro* exposure to concanavalin A (ConA) or lipopolysaccharide (LPS) in 21 pups ranging in age from 7 to 32 days old. All SSL pups responded to *in vitro* stimulation with ConA and LPS 055:B5 indicating peripheral T and B cells are capable of responding to an antigenic challenge. ConA- induced T cell proliferation decreased with age while there was no change in spontaneous proliferation of PBMC or B cells exposed to LPS. The decreases in total WBC, neutrophil counts and T cell proliferation indicates that SSL undergo a period of postnatal development in cell-mediated immune function which is comparatively longer than phocid pups and consistent with other otariids.

2.2 Introduction

The western population of Steller sea lions has declined by more than 80% since the 1970's (Loughlin, 1998; Sease *et al.*, 2001) and is listed as endangered under the Endangered Species Act. While the mechanistic cause of the decline is a matter of continuing debate (Springer *et al.*, 2003; Fritz and Hinckley, 2005; DeMaster *et al.*, 2006; Trites *et al.*, 2007; Atkinson *et al.*, 2008), studies have suggested a reduction in juvenile survival (York, 1994; Holmes and York, 2003). As a result, many studies have focused

[1]Keogh, M.J., J. M. Maniscalco, and S. Atkinson. 2010. Vet. Immunol. Immunopathol. 137:298-304.

on SSL pups and juveniles (Castellini *et al.*, 1993; Rea *et al.*, 1998; Brandon *et al.*, 2005; Myers *et al.*, 2010).

Hematology has been routinely used to assess the health of individuals and populations of SSL (Castellini *et al.*, 1993; Rea *et al.*, 1998). Hematocrit (Hct), hemoglobin (Hb), white blood cell (WBC) and platelet counts (Plt) have been used to assess nutritional status in pinnipeds (Thompson *et al.*, 1997; Rosen *et al.*, 2004; Trumble *et al.*, 2006). While red blood cell (RBC), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), Hct, and Hb decreased following birth, the same parameters increased to adult levels between 5 and 9 month in association with the development of diving ability and oxygen storage in SSL pups and juveniles (Richmond *et al.*, 2005).

In addition to hematology, PBMC proliferation assays are increasingly used to assess immuno-competence in pinnipeds (Swart *et al.*, 1993; Ross *et al.*, 1994; Swart *et al.*, 1994; Levin *et al.*, 2005; Mori *et al.*, 2006); however, few studies have assessed postnatal changes (Ross *et al.*, 1993; Ross *et al.*, 1994; Beckmen *et al.*, 1999; Kakuschke *et al.*, 2008). Ross *et al.*, (1993; 1994) concluded that harbor seal (HS; *Phoca vitulina*) pups have a functionally competent immune system at birth based upon functional changes during the 15 days following birth including: 1) a rapid increase in total IgG in serum, 2) *in vivo* antibody response to immunization, and 3) PBMC proliferation increases in response to *in vitro* mitogen stimulation. These conclusions were supported by the higher PBMC proliferation in HS pups compared to adults by Kakuschke *et al.*, (2008). The authors suggest these findings are due to unique life history traits in HS including long gestational period, large birth size, and short lactation period (Bowen *et al.*, 1992; Bowen *et al.*, 2001). Similarly, SSL have relatively long gestational periods and large pups when compared on a mass-specific basis of the female (Kovacs and Lavigne, 1992), although SSL have long periods of dependency with lactation lasting a year or more (Boness and Bowen, 1996). In contrast to the rapid increase in Ig in HS pups, the transfer of maternal antibodies, whether by placenta or colostrum, from adult northern fur seals (NFS; *Callorhinus ursinus*) to pups was low, despite adult Ig levels

similar to terrestrial mammals (Cavagnolo and Vedros, 1979). These findings were further supported following a *Klebsiella pneumonia* epizootic event in New Zealand sea lions (NZSL; *Phocarctos hookeri*). Castinel *et al.*, (2008) found that maternal antibodies for *K. pneumonia* were not passively transferred to pups and seroconversion occurring a minimum of 2 months after exposure. These findings suggest that unlike HS, the immune system of sea lions have a longer postnatal development period.

Capture and handling methods routinely used during blood collection in wild mammals can result in elevated cortisol concentrations (Engelhard *et al.*, 2002; Romero *et al.*, 2008), which may alter hematological parameters (Castellini *et al.*, 1996; Cattet *et al.*, 2003). Elevated cortisol concentrations have been shown to suppress PBMC proliferation in piglets (Westly and Kelley, 1984) while having no influence on lambs (Minton and Blecha, 1990) or calves (Manak, 1986). Given the inherent requirements of handling and the potential influence of cortisol on hematological components and *in vitro* PBMC proliferation, we quantified serum cortisol concentrations in an effort to assess the impact of sampling methods on these parameters.

The aim of the current study was to assess postnatal changes in circulating immune components and *in vitro* PBMC proliferation while accounting for potential effects of handling. Previous studies suggest that unlike HS, the immune system of sea lions may not be fully competent at birth (Ross *et al.*, 1993; Ross *et al.*, 1994). Therefore, we hypothesized that PBMC of SSL pups will proliferate when exposed to *in vitro* mitogen stimulation and that proliferation will be influenced by age as has been shown in other pinnipeds.

2.3 Materials and Methods

2.3.1 Study Site and Animals

Chiswell Island is home to a small SSL rookery located within the Alaska Maritime National Wildlife Refuge System, used by about 90 breeding animals producing up to 80 pups per year (Maniscalco *et al.*, 2006). The Chiswell Island rookery has been observed via remotely operated video monitoring equipment since 1998

(Maniscalco *et al.*, 2006). A total of 46 pups were measured, weighed, and blood sampled (Table 2.1). SSL pups were captured by corralling them in a natural rock formation on the rookery and later taking them to sampling stations individually. Body mass (BM) was

Table 2.1 Mean (± SD) for age, body mass (BM), standard length (SL), and axillary girth (AG) for SSL pups by sex and year.

		n	Age (day)	BM (kg)	SL (cm)	AG (cm)
June 30, 2005	Male	17	16.5 ± 5.2	31.5 ± 3.9	106.8 ± 4.0	74.1 ± 5.2
	Female	5	14.0 ± 5.6	28.0 ± 4.7	102.7 ± 4.9	71.4 ± 6.4
July 3, 2007	Male	10	23.3 ± 7.1	34.6 ± 4.5	110.4 ± 4.4	79.8 ± 4.4
	Female	5	14.1 ± 5.0	26.9 ± 6.0	101.8 ± 5.8	71.7 ± 6.8
July 1, 2008	Male	4	18.7 ± 10.4	32.3 ± 7.9	106.5 ± 8.7	75.3 ± 10.0
	Female	5	13.1 ± 3.9	25.6 ± 6.7	101.8 ± 2.8	72.3 ± 9.1
All	Male	31	18.9 ± 7.1	32.6 ± 4.7	107.9 ± 5.0	76.1 ± 6.8
	Female	15	13.8 ± 4.5	26.8 ± 4.4	102.1 ± 4.3	70.7 ± 5.7

measured to the nearest tenth of a kilogram using a hanging electronic scale (FWC series 7, FlexWeigh, Santa Rosa, CA). Standard length (SL) was measured as a straight line from tip-of-nose to tip-of-tail while the pup was lying on a straight board. Axillary girth (AG) was measured using a tape measure after the pup had exhaled. Pups (n=43) were permanently marked by hot iron branding (Merrick *et al.*, 1995) under isoflurane (USP; Halocarbon Industries, River Edge, NJ) anesthesia (Heath *et al.*, 1997) with the remaining three pups having flipper tags attached in the axillary area of both fore-flippers. Branding pups in conjunction with the remote video monitoring program allowed for the identification of mother-pup pairs and determination of pup ages (to within ± 4 hrs) by association with naturally marked females that were tracked from the time they gave birth. Pups ranged in age from 5 to 38 days old at the time of sampling.

Blood samples (< 16 ml) were collected using standard aseptic techniques from the caudal gluteal vein with an 18 gauge needle directly into Vacuette® blood collection tubes. Samples were collected in collaboration with other research and branding efforts. In 2005 and 2008 pups were anesthetized with isoflurane (ISO) prior to blood collection

and pups were physically restrained (PR) in 2007 during blood collection. EDTA-treated blood tubes were collected for CBC counts including total and differential WBC counts. Serum separator tubes were collected and serum was stored at -80 °C until cortisol was quantified. Sodium heparin-treated tubes were collected in 2007 and 2008 for isolation of PBMC from 21 of the pups (10 female, 11 male). Blood tubes were kept upright and chilled until further processing in the lab (< 12 hrs).

2.3.2 Complete Blood Cell Count

CBC counts were determined using the Heska® CBC-Diff Veterinary Hematological System (Heska® Corporation, Loveland, CO). Total WBC, Hct, RBC, Hb, and Plt were measured and used to calculate the MCV, MCH, and MCHC. Blood smears were made from whole EDTA blood and stained with Wright-Giemsa (Dip Quick Stain, Jorgensen Laboratories, Loveland, CO). WBC differentials, including neutrophils, lymphocytes, monocytes and eosinophils were counted manually and reported as percentages and absolute values of differentials (cells/μl).

2.3.3 Cortisol Radioimmunoassay

Cortisol was quantified using a solid-phase radioimmunoassay (RIA) (Siemens, Los Angeles, CA) previously validated for use with unextracted SSL serum (Mashburn and Atkinson, 2004). The RIA was performed per manufacturer instructions with the exception that all volumes were halved and run in duplicate. Inter-assay coefficients of variation for two assay controls were 5.6 and 10.0%. Intra-assay coefficients of variation were < 5.0 % and assay sensitivity was 2.0 ng/ml.

2.3.4 PBMC Isolation and Proliferation

Sodium heparin blood tubes were centrifuged at 200 x g for 10 min and the buffy coat was re-suspended in freezing media comprised of RPMI 1640, 20% FBS (Hyclone, Thermo Fisher Scientific Inc., Logan, UT), and 10% DMSO (Sigma Chemical Co., St. Louis, MO). Cryopreserved WBC were thawed and washed twice with PBS (without

Ca^{2+}/Mg^{2+}) and re-suspended in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). PBMC were isolated by density gradient centrifugation (Histopaque 1077; MP Biomedical, Solon, OH) for 20 min at 720 x g. The PBMC containing band was isolated, washed twice, and re-suspended in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml), 1 mM sodium pyruvate, 100 µM nonessential amino acids, 25 mM hepes and 0.05 mM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). All cell culture media and supplements, except for FBS, DMSO and 2-mercaptoethanol, were purchased from Gibco, Invitrogen Corp., Carlsbad, CA. Viability was >90% and assessed using the exclusion dye trypan blue (Sigma Chemical Co., St. Louis, MO).

PBMC were plated (2.0×10^5 cells/well) in 96-well flat-bottom tissue-culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) and their proliferation assessed following exposure to one of two mitogens (ConA or LPS 055:B5; Sigma Aldrich, St. Louis, MO). PBMC were cultured at 37°C with 5% CO_2 in triplicate for each animal in each of the following treatment groups; 1) unexposed control, 2) ConA suboptimal (0.1 µg/ml), 3) ConA optimal (1.0 µg/ml), 4) LPS suboptimal (1.0 µg/ml) and 5) LPS optimal (100 µg/ml). ConA is a plant-derived mitogen and preferentially stimulates T cells (Barta and Barta, 1993) while the LPS from *Escherichia coli* is a B cell activator (Wechsler-Reya and Monroe, 1996). The use of suboptimal and optimal mitogen concentrations have been used in previous pinniped studies (Levin *et al.*, 2005; Mori *et al.*, 2006). Mitogens were reconstituted in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). These conditions are similar to those previously used for pinnipeds (Levin *et al.*, 2005; Mori *et al.*, 2006).

All PBMC were assessed in two assays with PBMC collected from a captive adult male SSL as an inter-assay control. PBMC were incubated for 48 h after which 20 µl of bromodioxyuridine (BrdU) was added to a final concentration of 10 µM BrdU and incubated for an additional 18 h for a total incubation period of 66 h. Proliferation was assessed with the Cell Proliferation Biotrack ELISA System version 2 (GE Healthcare,

Piscataway, NJ) per manufacturer's instructions using Dynatech MRX Revelation microplate reader (Dynatech Laboratories, Inc, Chantilly, VA) at 450 nm with a reference wavelength of 650 nm. Data are presented as mean of the triplicates for optical density (OD) with standard deviation and as a stimulation index (SI; mean OD of cells exposed to mitogen/mean OD of cells in media only).

2.3.5 Statistical Analysis

Data were analyzed with Systat 10 (Systat Software, Inc, Point Richmond, CA). The best model for each parameter was selected using a stepwise general linear model with an iterative process of comparing the full mixed effects model, which included the categorical variables of sex and anesthesia, age as a continuous variable, and all interaction terms. The full model was compared to reduced models, which included only statistically significant grouping variables and interactions. Three of the 13 hematological parameters (MCV, MCH, MCHC) showed differences between PR and ISO; however, differences were less than 5% and therefore the hematological parameters were not separated for further analysis. To evaluate the effect of pup handling, serum cortisol concentrations were regressed against either the time elapsed from the initial arrival on the rookery to the time when the individual pup was removed from the corral and taken for blood collection or the immune components measured in the current study. Values were considered statistically significant if $p \leq 0.05$.

2.4 Results and Discussion

The ability to acquire blood samples from wild SSL pups of known age is a unique opportunity, which enabled us to assess postnatal changes in circulating immune components and PBMC proliferation. Based on BM and morphometrics all pups sampled were in good body condition for their respective age and sex. There was no effect of sex ($p=0.338$), anesthesia ($p=0.171$) or age ($p=0.854$) on circulating cortisol levels. Cortisol concentrations decreased when regressed against the elapsed time between arrival on the rookery and removal of pup from the holding corral for blood collection ($p=0.009$),

indicating that the pups were affected by the initial disturbance of the rookery. However, no significant effects of sex, anesthesia or serum cortisol concentrations ($p>0.082$) were detected in unstimulated or mitogen stimulated PBMC.

Proliferation of unstimulated PBMC and PBMC exposed to LPS and the suboptimal ConA did not change with age (Table 2.2). In piglets (Hoskinson *et al.*, 1990)

Table 2.2 Mean (± SD) and range of cortisol, hematology and PBMC proliferation from SSL pups. Age effect was significant at $p<0.05$.

Parameter	n	Means			Ranges			Age
Cortisol (ng/ml)	46	136.8	±	32.4	86.7	–	216.0	p=0.854
WBC ($10^3/\mu l$)	42	13.1	±	2.8	7.3	–	19.6	p<0.001
Neutrophil ($10^3/\mu l$)	42	8.60	±	2.54	4.36	–	16.97	p=0.001
(% of WBC)	42	64.9	±	9.2	45.0	–	84.0	p=0.076
Lymphocyte ($10^3/\mu l$)	42	2.55	±	1.18	1.12	–	5.63	p=0.871
(% of WBC)	42	19.7	±	8.3	8.0	–	42.0	p=0.128
Monocytes ($10^3/\mu l$)	42	1.54	±	0.87	0.0	–	3.8	p=0.698
(% of WBC)	42	11.83	±	6.35	0.0	–	24.0	p=0.481
Eosinophil ($10^3/\mu l$)	42	0.44	±	0.38	0.0	–	1.16	p=0.635
(% of WBC)	42	3.5	±	3.1	0.0	–	10.0	p=0.467
N:L ratio	42	4.06	±	2.16	1.07	–	9.75	p=0.042
Hct (%)	42	34.6	±	3.1	29.7	–	43.8	p=0.142
MCV (fl)	42	100.0	±	3.6	93.4	–	108.2	p=0.594
RBC ($10^6/\mu l$)	42	4.3	±	3.5	3.0	–	4.3	p=0.175
Hb (g/dl)	42	12.5	±	1.1	10.7	–	15.0	p=0.229
MCH (pg)	42	36.3	±	1.5	32.5	–	40.0	p=0.416
MCHC (g/dl)	42	36.3	±	1.8	34.0	–	39.4	p=0.774
PLT ($10^3/\mu l$)	42	367.9	±	120.1	93.0	–	639.0	p=0.496
Unstimulated (OD)	21	0.300	±	0.029	0.240	–	0.350	p=0.942
ConA								
0.1 µg/ml (OD)	21	0.313	±	0.040	0.240	–	0.370	p=0.137
0.1 µg/ml (SI)	21	1.05	±	0.11	0.890	–	1.280	p=0.137
1.0 µg/ml (OD)	21	0.842	±	0.784	0.310	–	3.130	p=0.039
1.0 µg/ml (SI)	21	6.10	±	2.49	1.330	–	10.440	p=0.038
LPS 055:B5								
1.0 µg/ml (OD)	21	0.408	±	0.131	0.238	–	0.661	p=0.728
1.0 µg/ml (SI)	21	1.360	±	0.410	0.900	–	2.10	p=0.670
100 µg/ml (OD)	21	0.636	±	0.278	0.238	–	1.210	p=0.309
100 µg/ml (SI)	21	2.080	±	0.850	0.920	–	3.740	p=0.293

and dogs (Toman *et al.*, 2002; Faldyna *et al.*, 2005) high spontaneous proliferation of PBMC were observed during the first week after birth and subsequently decreased within four to six weeks. The cause of the high spontaneous proliferation was unclear but may be due to PBMC being immature or in an activated state (Hoskinson *et al.*, 1990). In the present study, PBMC proliferated when exposed *in vitro* to ConA and LPS compared to unstimulated PBMC, suggesting that peripheral T and B cells are capable of responding to an antigenic challenge as early as 5 days following birth. Further, T cell proliferation decreased with age when exposed to the optimal ConA (Table 2.2, Figure 2.1). Similar

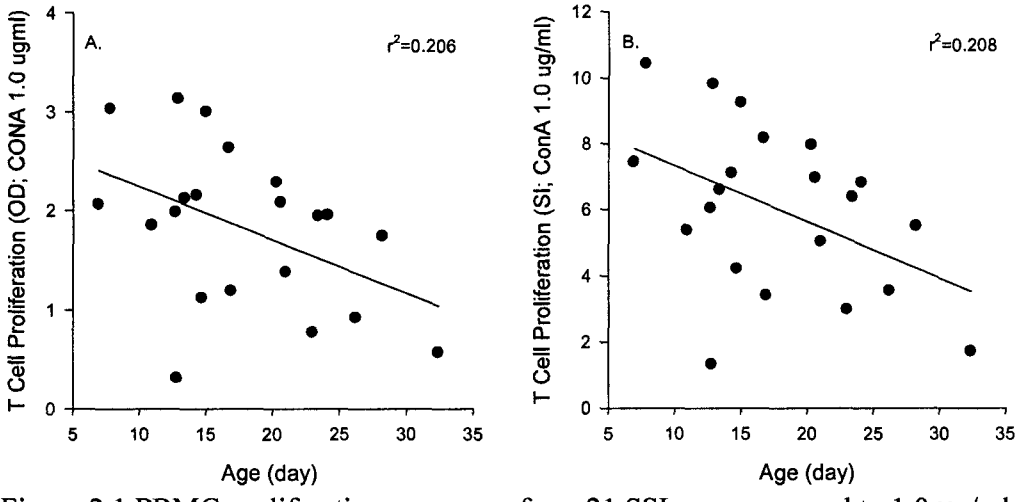


Figure 2.1 PBMC proliferation responses from 21 SSL pups exposed to 1.0 µg/ml ConA. Results presented as (A) absorbency (OD) and (B) stimulation index (SI). Significant ($p \leq 0.05$) trend lines are presented.

decreases in T cell proliferation during the first month of life have been observed in dogs followed by a gradual increase after 6 weeks, remaining below proliferation levels of adult dogs until at least 3 months of age (Gerber and Brown, 1974; Toman *et al.*, 2002). Similarly, an initial decrease in proliferation was observed in piglets (Hoskinson *et al.*, 1990), rabbits (Jeklova *et al.*, 2007), and NFS pups (Beckmen, 1999).

Beckmen (1999) reported a decrease in T cell proliferation exposed to ConA in NFS sampled as neonates (<7 days) and again at an estimated 29-51 days old. Moreover,

NFS pups born to older females (43%) developed adequate antibody titers when vaccinated with tetanus toxoid compared to only 5% of pups born to young females (Beckmen *et al.*, 2003). The low humoral immune response in pups of young females was associated with milk comparatively lower in fat (Beckmen *et al.*, 1999) and higher organochlorine (OC) levels resulting in the higher OG levels in the blood of NFS pups (Beckmen *et al.*, 1999; Beckmen *et al.*, 2003).

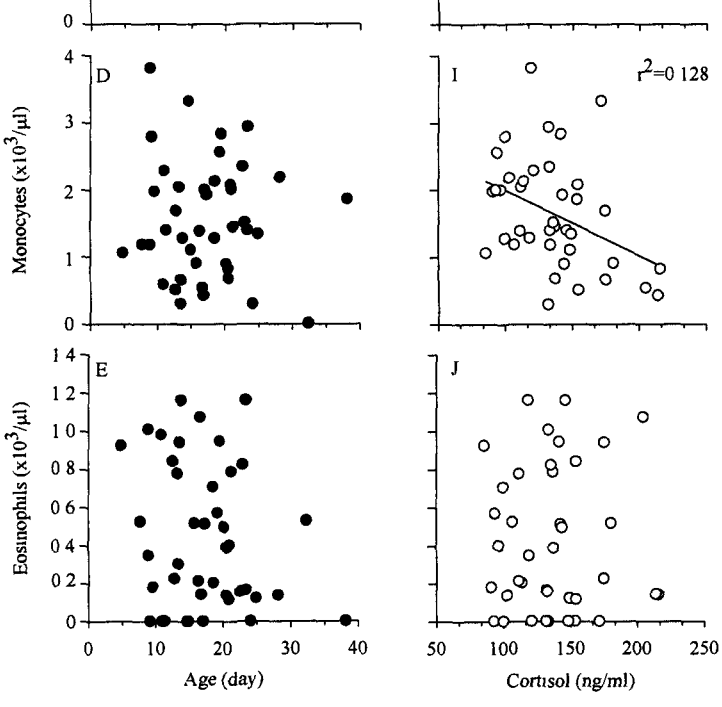
Development of antibodies against tetanus is dependent on T cells (Willcox, 1975). Decrease in T cell proliferation following birth observed in the current study and in NFS (Beckmen, 1999) suggests that age at time of vaccination is another factor contributing to the low humoral immune response observed by Beckmen *et al.*, (2003). Only 23% of the vaccinated NFS pups showed a 1.7-fold or greater increase in serum tetanus antibody, indicating a low humoral response in both the number of seroconverted individuals and in the low level of tetanus antibodies in pups considered to have achieved seroconversion (Beckmen *et al.*, 2003). These findings are consistent with observations in NZSL following a *K. pneumonia* epizootic event (Castinel *et al.*, 2008). In our study, SSL pups had a decrease in T cell proliferation comparable to changes observed in NFS (Beckmen, 1999) suggesting that SSL pups would have a similarly low or delayed humoral immune response as observed in other otariid pups (Beckmen *et al.*, 2003; Castinel *et al.*, 2008).

There was not a significant effect of sex or anesthesia on total or differential WBC counts, while monocyte counts ($p= 0.020$, Figure 2.2I) decreased in association with increasing cortisol concentrations. However, cortisol concentrations ($p>0.059$) did not affect total WBC, neutrophil, lymphocyte, eosinophil counts or CBC count parameters including Hct, RBC, Hb, or PLT, suggesting handling of pups did not alter circulating immune components. Total WBC and neutrophil counts decreased while there was no change in lymphocyte, monocyte, or eosinophil counts with age (Table 2.2, Figure 2.2). The neutrophil to lymphocyte ratio also decreased with age. The decreases in total WBC and neutrophil to lymphocyte ratio were driven by changes in neutrophils. In HS pups, neutrophils also decreased but no change was observed in total WBC counts

due to a concurrent increase in lymphocytes and monocytes (Ross *et al.*, 1994). Hasselmeier *et al.*, (2008) also found higher levels of lymphocytes and monocytes in pups compared to older HS. Mean total WBC count in the current study was higher than reported values for SSL pups and juveniles (Mellish *et al.*, 2006). The higher WBC count in our study is likely due to pup age. Mellish *et al.* (2006) sampled older SSL pups (6 to 10 months) and juveniles (1 to 3 y) concluding that pups had significantly lower WBC counts than juveniles. Taken with the WBC counts in the current study, young SSL pups have a higher WBC followed by a postnatal decrease remaining below the levels observed in juveniles. Differential WBC counts in our study were within ranges reported for healthy adult California sea lions, *Zalophus californianus* (Roletto, 1993) and SSL pups (Bishop and Morado, 1995). Specifically, Bishop and Morado (1995) found similar percentage of neutrophil (67.7 ± 6.6), lymphocyte (15.1 ± 5.6), monocyte (11.5 ± 4.8) , and eosinophils (3.5 ± 2.8) though the mean total WBC was lower than in our study. Difference in total WBC count is likely explained by methodological differences between studies, total WBC counts were based upon estimated WBC density in the blood smear slides in the Bishop and Morado (1995) study.

This study adds to the limited knowledge of the postnatal changes in SSL pups. The decreases in total WBC, neutrophil counts and T cell proliferation indicates that SSL undergo a period of postnatal development in cell-mediated immune function, which is comparatively longer than in phocid pups and consistent with other otariids. The postnatal changes in SSL pups may be associated with differences in disease susceptibility between pups and adults. Future studies are needed to assess when pups or juveniles reach immuno-competence and if the development of immune function is associated with disease susceptibility contributing to the reduction in juvenile survival suggested in some SSL populations (York, 1994; Holmes and York, 2003). Given the unique life history traits of pinnipeds, these findings are relevant for comparative immunology and have implications for the interpretation of hematological parameters such as total and differential WBC counts in studies conducted on free-ranging SSL pups.

Figure 2.2 (A) Total WBC counts ($10^3/\mu l$) from 42 pups during the 38 days following birth and differential counts for (B) neutrophils ($10^3/\mu l$); (C) lymphocytes ($10^3/\mu l$); (D) monocytes ($10^3/\mu l$); and (E) eosinophils ($10^3/\mu l$). (F) Total WBC counts ($10^3/\mu l$) (G) neutrophils ($10^3/\mu l$); (H) lymphocytes ($10^3/\mu l$); (I) monocytes ($10^3/\mu l$); and (J) eosinophils ($10^3/\mu l$) are also presented against level of circulating cortisol (ng/ml). Significant ($p \leq 0.05$) trend lines are presented.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I
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Monocytes (x10^3/μl)
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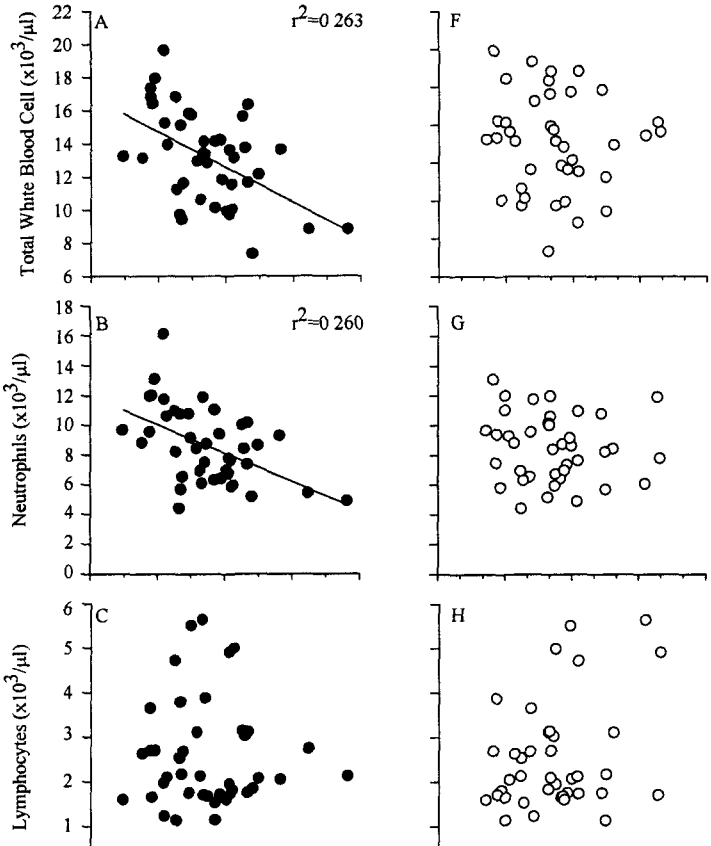
Eosinophils (x10^3/μl)
Age (day)
Cortisol (ng/ml)


A
r^2=0 263
F
Total White Blood Cell (x10^3/µl)
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20
18
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14
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B
r^2=0 260
G
Neutrophils (x10^3/µl)
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10
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6
4
2
0
C
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Lymphocytes (x10^3/µl)
6
5
4
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1

2.5 Acknowledgements

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Chapter 3:

Body condition and endocrine profiles of healthy Steller sea lion (*Eumetopias jubatus*) pups during the early postnatal period[1]

3.1 Abstract

Body condition indices have been useful in accessing the health in domestic livestock and free ranging populations of terrestrial mammals. Given the high energy demand and rapid growth during the postnatal period of Steller sea lion (SSL; *Eumetopias jubatus*) pups, body condition was expected to be related to circulating concentrations of a suite hormones (cortisol, aldosterone, thyroxine , triiodothyronine, leptin) associated with changes in body composition over time in developing pinnipeds. Blood samples were collected from free ranging SSL pups of known ages and sex. A body condition index (BCI) previously developed for SSL pups based on mass and length relationship was applied to 61 SSL pups ranging in age from 5 to 38 days old. However, no relationship between BCI and circulating concentration of hormones was detected. Circulating concentrations of cortisol, total and free thyroxine, and total triiodothyronine decreased when regressed against the elapsed time between researchers' arrival on the rookery and removal of pup from the holding corral for blood collection. While the overall variation attributed to the rookery disturbance was low ($r^2<0.293$), it may be of significance for future studies on free ranging pinnipeds. SSL pups sampled in the present study were larger on average than predicted by growth rates previously reported for SSL pups. These findings suggest that pups sampled in the present study are from a healthy population of SSL. This study adds to the limited knowledge of the postnatal changes in free ranging SSL pups by providing concentration ranges of several hormones from an apparently healthy population.

[1]Keogh, M. J., S. Atkinson, J. M. Maniscalco. J. Comp. Physiol. B. *in submission*

3.2 Introduction

Body condition indices (BCI) have been applied in livestock to assess health and production measures (Caldeira et al. 2007) and to free ranging populations of terrestrial mammals (Cattet et al. 2002). Increasingly there has been an interest in quantifying body condition in pinnipeds as a measure of health and fitness (Arnould and Hindell 1999; Arnould and Warneke 2002; Castellini and Calkins 1993; Jonker and Trites 2000; Pitcher et al. 2000; Rea 2002; Trites and Jonker 2000). BCI for Steller sea lions (SSL; *Eumetopias jubatus*) have been derived from a combination of mass, length and blubber thickness (Pitcher et al. 2000; Rea 2002; Trites and Jonker 2000). BCI previously developed for SSL pups based on mass and length relationship were able to distinguish between dead pups with and without fat stores (Trites and Jonker 2000); however, how the BCI relate to circulating hormone concentrations has not been explored in SSL pups.

Changes in body condition or mass have been associated with changes in circulating cortisol, thyroid hormones and leptin in several species of terrestrial mammals (Barboza et al. 2004; Buff et al. 2002; Caldeira et al. 2007; Concannon et al. 2001) and pinnipeds (Jeanniard du Dot et al. 2009; Ortiz et al. 2003). Decreases in lipid stores and mass were associated with an increase in circulating cortisol concentration and decrease in total thyroxine and triiodothyronine concentrations in SSL (Jeanniard du Dot et al. 2009; Rosen and Kumagai 2008). These previous studies were based on longitudinal sampling during periods of experimental food restriction or fasting in juvenile and sub-adult SSL. Given the high energy demand and rapid growth (0.23–0.48 kg/day) during the postnatal period of SSL pups (Brandon et al. 2005), it is reasonable to expect an association between body condition and circulating concentrations of hormones associated with nutritional status and fat mass (leptin), lipid and water metabolism (cortisol and aldosterone), and growth and metabolism (thyroxine and triiodothyronine).

Currently two distinct population segments (DPS) of SSL are recognized within US waters: the eastern and western DPS. The western DPS is listed as endangered under the Endangered Species Act (U.S. Federal Register 62:30772–30773) having declined by more than 80% since the 1970's (Loughlin 1998; Sease et al. 2001). The causes of the

population decline and failure to recover have been the focus of continuing research and debate (Atkinson et al. 2008; DeMaster et al. 2006; Fritz and Hinckley 2005; Springer et al. 2003; Trites et al. 2007), predominately focusing on one or a combination of several factors including fisheries and interspecies competition for resources leading to a reduction in quantity or quality of food, human disturbance, predation, and exposure to contaminants. However, there remains no clear reason for the observed decline, which places an even greater emphasis on assessing the physiological factors (i.e. endocrine profiles) that impact body condition, and thus, health of this vulnerable species.

The present study sampled pups from a SSL rookery located on the northwestern shore of Chiswell Island in the eastern Gulf of Alaska within the western DPS. Prior to the western DPS decline, Chiswell Island supported a dramatically larger population of approximately 2,000 SSL (Mathisen and Lopp 1963); currently about 90 breeding animals use the rookery producing up to 80 pups annually (Maniscalco et al. 2006). Blood samples were collected from SSL pups of known ages, sex and body condition providing an opportunity to measure circulating hormones during the early postnatal period, and to determine if these hormones could provide predictable markers for evaluating the body condition of pups to better assess the health of the population of SSL. Therefore, the aims of the present study were to quantify circulating concentrations of cortisol, aldosterone, total and free thyroxine (T_4), total triiodothyronine (T_3), and leptin in SSL pups, and to determine if these hormones were associated with body condition. We hypothesized circulating concentrations of cortisol and aldosterone to decrease while leptin and thyroid hormones increase with increasing BCI. Further, capture and handling methods routinely used during blood collection in wild mammals can result in elevated circulating concentrations of cortisol (Engelhard et al. 2002; Romero 2002), aldosterone (Thomson and Geraci 1986), and thyroid hormones (Gartner et al. 1980; St. Aubin and Geraci 1988). Therefore, given the inherent requirements of handling necessary for sampling free ranging pinnipeds, we assessed the impact of the rookery disturbance on the hormones quantified in the present study.

3.3 Materials and Methods

3.3.1 Animals and Body Condition

A total of 61 randomly selected pups (male=37, female=24) were measured, weighed, and blood sampled on June 30, 2005; July 3, 2007 and July 1, 2008 (Table 1). SSL pups were captured after clearing the rookery of all adult SSL and corralling the pups in a natural rock formation on the rookery. Following capture, pups were individually taken to blood sampling stations. The time from corralling to removal for sampling ranged from 1 hr and 20 min to 8 hrs and 40 min. Body mass was measured to the nearest tenth of a kilogram using a hanging electronic scale (FWC series 7, FlexWeigh, Santa Rosa, CA), standard length was measured as a straight line from tip-of-nose to tip-of-tail while the pup was lying on a straight board with its ventral surface down, and axillary girth was measured using a tape measure after the pup exhaled. BCI were calculated by applying the stoutness-index [body mass/ (-63.88 + 0.8966*standard length)] proposed by Trites and Jonker (2000).

Pups were marked by either branding (Merrick et al. 1995) or flipper-tagging for further identification to determine age. Marking pups in conjunction with the remote video monitoring program allowed for the identification of mother-pup pairs and determination of pup ages (to within ± 4 hrs) by association with naturally marked females that were tracked from the time they gave birth. Pups ranged in age from 5 to 38 days old at the time of blood collection. All SSL were continuously observed during daylight hours by the remote video camera monitoring system at the Alaska SeaLife Center (Seward, AK) throughout the entire breeding season (Maniscalco et al. 2006). Pups ranged in age from 5 to 38 days at the time of blood collection.

3.3.2 Blood Collection

Blood samples (< 18 ml) were collected using standard aseptic techniques from the caudal gluteal vein directly into Vacuette® serum separator blood collection tubes. In 2005 and 2008 pups were anesthetized under isoflurane (USP; Halocarbon Industries, River Edge, NJ) (Heath et al. 1997) prior to blood collection, and in 2007, pups were

physically restrained during blood collection. Serum separator tubes were kept upright and on ice until further processing in the laboratory (< 12 hrs). Serum was thereafter stored at -80°C until analysis.

3.3.3 Hormone Radioimmunoassays

Commercially available radioimmunoassay (RIA) kits were used to quantify hormone concentrations. All samples were run in duplicate per manufacturer instructions except where noted below, and with the exception that all volumes were halved. Solid-phase RIA kits (Siemens, Los Angeles, CA) were used for quantifying aldosterone, cortisol, and thyroid hormones. Cortisol, thyroid hormones (total T_3, total and free T_4), and leptin were previously validated in our laboratory (Mashburn and Atkinson 2004, 2008; Myers et al. 2006) and aldosterone was validated in the present study. Pools of male and female SSL serum were serially diluted to determine degree of parallelism to the standard curve. Recovery of added aldosterone (25–1200 pg/ml) for males ($y=-20.474x + 161.79$, $r^2=0.991$) and for females ($y=-20.803x + 164.39$; $r^2=0.997$) was determined. Leptin was assayed with a double-antibody multi-species leptin RIA kit (Linco Research, St. Charles, MO) previously validated with the following additional modifications to the manufacturer's protocol: the incubation period with primary antibody was doubled to 48 hr and buffer was not added to samples during incubation with the primary antibody (Mashburn and Atkinson 2008). Inter-assay and intra-assay coefficients of variation were <10.0% for each hormone. Assay sensitivities were as follows: cortisol, 2.0 ng/ml; aldosterone, 11.0 pg/ml; total T_3, 0.07 ng/ml; total T_4, 2.5 ng/ml; and free T_4, 0.1 pg/ml; leptin, 0.5 ng/ml human equivalent (HE).

3.3.4 Statistical Analysis

The best model for each hormone was selected using a stepwise general linear model with an iterative process of comparing the full mixed effects model, which included the categorical variables sex and anesthesia /physical restraint, with BCI or age as a continuous variable, and all interaction terms. The full model was compared to

reduced models, which included only variables and interactions with a $p \leq 0.100$. If anesthesia /physical restraint were identified as a significant factor, then pups under anesthesia or physical restraint were analyzed separately for effects of sex, age or BCI. Aldosterone concentrations were log transformed prior to statistical analysis to normalize the data. Mean values are reported ± standard deviation (SD) and results were considered statistically significant if $p \leq 0.050$. To evaluate the effect of handling on pups, serum hormone concentrations measured in the present study were regressed against the time elapsed from the initial arrival on the rookery to the time when the individual pup was removed from the corral and taken for blood collection. Data were analyzed with Systat 10 (Systat Software, Inc, Point Richmond, CA).

3.4 Results

3.4.1 Age and Body Mass

BCI was not related to pup age ($p=0.216$) nor was there an effect of sex ($p=0.647$). Female pups were smaller than males in body mass ($p< 0.001$), standard length ($p< 0.001$), and axillary girth ($p=0.015$). SSL pups increased in body mass ($p<0.001$), standard length ($p<0.001$), and axillary girth ($p<0.001$) with age (Fig. 1) though female pups (14.7 ± 4.9 days) were on average younger than male pups (18.4 ± 7.2 day) ($p=0.025$).

3.4.2 Hormonal Profiles and Relationships

Male pups (368.1 ± 253.3 pg/ml) had higher aldosterone concentrations ($p=0.015$) (Fig. 2a) than females (357.0 ± 301.0 pg/ml) and there was a significant interaction between sex and BCI ($p=0.013$). The difference between male (323.5 ± 143.5 pg/ml) and female (276.5 ± 111.9 pg/ml) pups remained significant even when the outliers were removed ($p=0.011$). No significant effect of age or anesthesia /physical restraint was detected. Nor were aldosterone concentrations effected by the elapsed time between arrival on the rookery and blood collection. Circulating cortisol concentrations were higher in female (151.4 ± 45.1 ng/ml) than male (129.8 ± 34.0 ng/ml) pups ($p=0.037$)

(Fig. 2b) but were not affected by anesthesia /physical restraint, BCI or age. Cortisol concentrations decreased when regressed against the elapsed time between researchers arrival on the rookery and removal of pup from the holding corral for blood collection ($p<0.001$) (Fig. 3a).

Leptin (ng/ml HE) serum levels were significantly higher ($p= 0.009$) in PR (2.0 ± 0.4 ng/ml HE) than pups under ISO (1.7 ± 0.4 ng/ml HE) during blood collection. When pups under anesthesia or physical restraint were analyzed separately, there was no effect of BCI, sex or age on circulating leptin concentrations.

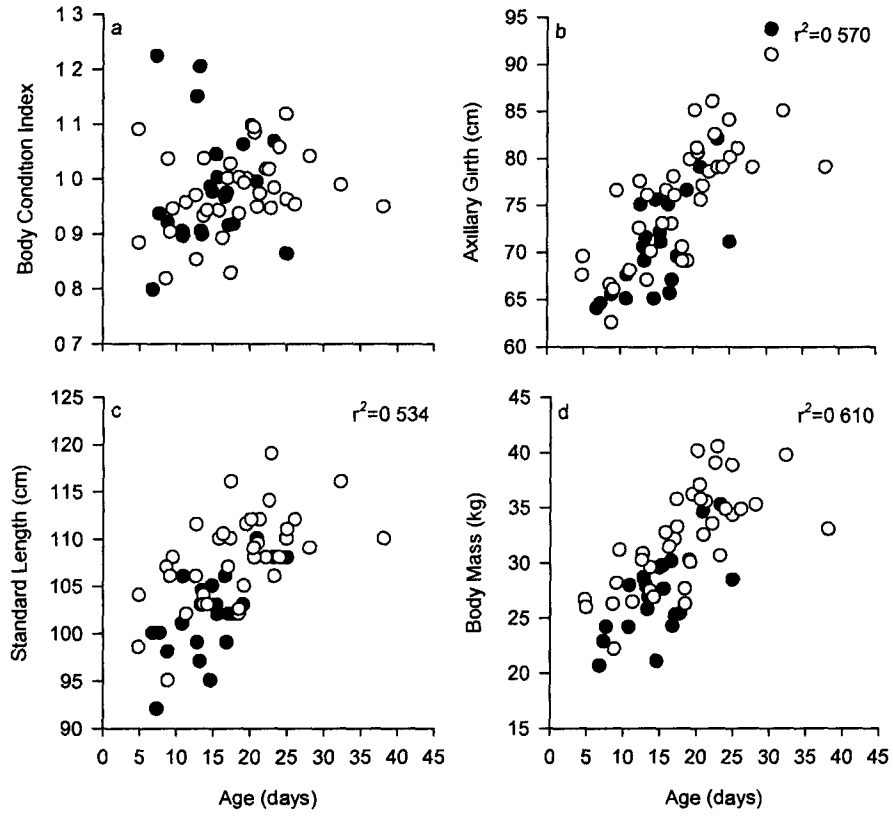

Fig. 3.1. A total of 61 pups (male=37, female=24) were sampled during 2005, 2007, and 2008. Morphometerics for individual pups at age are presented in (a) body condition index, (b) axillary girth, (c) standard length, and (d) body mass. Female pups are represented by (•) and male pups are by (○).

Table 3.1 Mean (± SD) for age, body mass, standard length, and axillary girth for SSL pups by year and sex.

		n	Age (day)	n	body mass (kg)	n	standard length(cm)	n	axillary girth (cm)
2005	Male	20	16.2 ± 5.3	20	31.2 ± 3.9	20	106.7 ± 3.7	20	73.6 ± 5.4
	Female	8	15.1 ± 4.5	8	27.5 ± 3.8	8	102.6 ± 3.7	8	70.7 ± 5.1
2007	Male	13	21.6 ± 8.1	13	33.8 ± 4.5	13	110.4 ± 4.5	13	78.7 ± 4.9
	Female	8	16.3 ± 5.5	9	27.9 ± 4.5	9	102.8 ± 4.6	9	72.5 ± 5.2
2008	Male	4	18.6 ± 10.4	4	32.2 ± 7.9	4	106.5 ± 8.7	4	75.2 ± 10.0
	Female	6	12.2 ± 4.2	7	24.3 ± 3.2	7	99.0 ± 5.3	6	66.7 ± 4.8
All	Male	37	18.3 ± 7.2	37	32.2 ± 4.6	37	108.0 ± 4.9	37	75.6 ± 6.1
	Female	22	14.7 ± 4.9	24	26.7 ± 4.1	24	101.6 ± 4.7	23	70.3 ± 5.4

* note: Age could not be determined from one female pup from 2007 and 2008 each.

There was no detectable effect of BCI, sex or ISO/PR on circulating total T_4 or free T_4 in SSL pups. Circulating total T_4 ($p= 0.015$) and free T_4 ($p=0.031$) decreased with pup age (Fig. 3.4). Pups under anesthesia (0.6 ± 0.1 pg/ml) had higher ($p=0.001$) total T_3 compared to pups under physical restraint (0.5 ± 0.1 pg/ml) during blood collection. When compared separately there was no significant effect of sex, BCI or age in total T_3. Thyroid hormones significantly decreased when regressed against the elapsed time from the initial arrival of researchers on the rookery to the time when the individual pup was removed from the corral and taken for blood collection (Fig. 3.3) including total T_4 ($p=0.008$), total T_3 ($p=0.012$), and free T_4 ($p<0.001$).

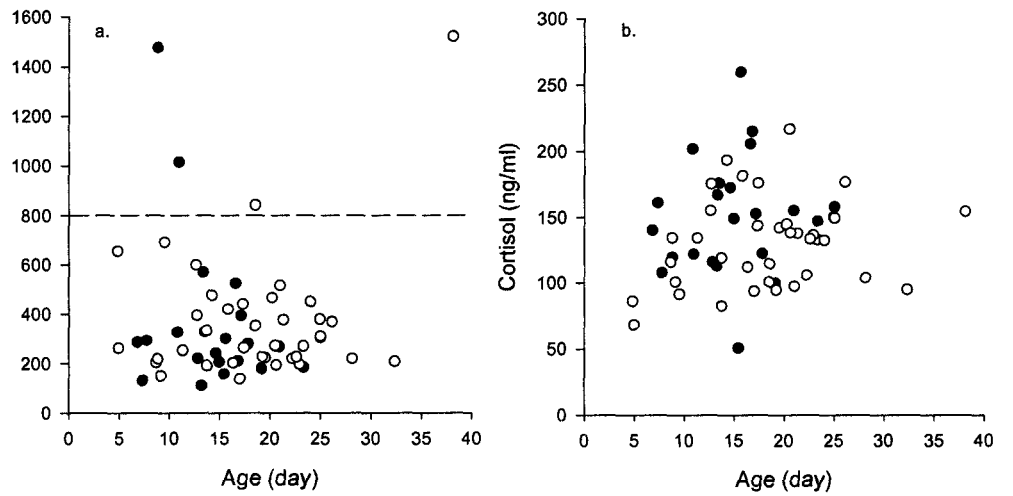

Fig. 3.2 (a) Aldosterone concentrations (pg/ml) and (b) cortisol concentrations (ng/ml) from female (•) and male (○) SSL pups. Dashed line separates the pups with greater than 2 SD of the mean for male and female pups for aldosterone concentration (pg/ml).

Table 3.2 Mean (± SD) for cortisol, aldosterone, total and free thyroxine (T_4), total triiodothyronine (T_3), and leptin concentrations for all 61 SSL pups and separated by sex (female=24, male=37).

	All pups Mean ± SD			Females Mean ± SD			Males Mean ± SD		
cortisol (ng/ml)*	138.3	±	39.8	151.4	±	45.1	129.8	±	33.9
aldosterone (pg/ml)*	364.2	±	270.6	357.0	±	300.9	368.8	±	253.3
total T_4 (ng/ml)	19.5	±	5.3	20.0	±	5.3	19.2	±	5.3
total T_3 (ng/ml)	0.6	±	0.1	0.6	±	0.2	0.5	±	0.1
free T_4 (pg/ml)	6.1	±	2.1	6.1	±	1.6	6.1	±	2.3
total T_4:free T_4	3.5	±	1.3	3.4	±	1.2	3.6	±	1.3
leptin (ng/ml HE)	1.9	±	0.4	1.9	±	0.4	1.7	±	0.4

*Statistically different between male and female pups at $p<0.050$.

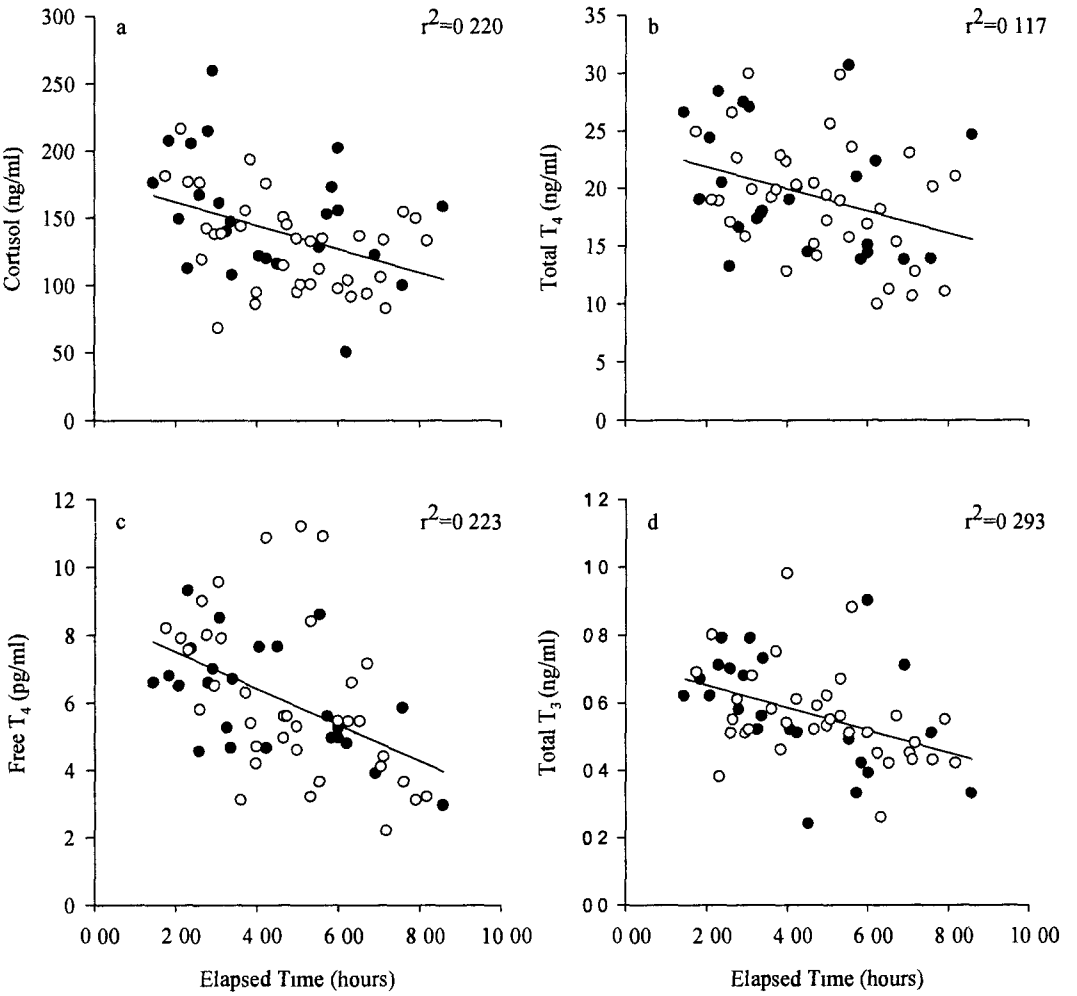

Fig 3.3 Circulating concentrations of (a) cortisol (ng/ml); (b) total T_4 (ng/ml); (c) free T_4 (pg/ml); (d) total T_3 (ng/ml) from female (●) and male (○) SSL pups regressed against the elapsed time from the initial arrival on the rookery to the time when individual pups were removed from the corral for blood collection. Female pups are represented by filled circles, males are by hollow circles. Significant ($p \leq 0.05$) trend lines are presented.

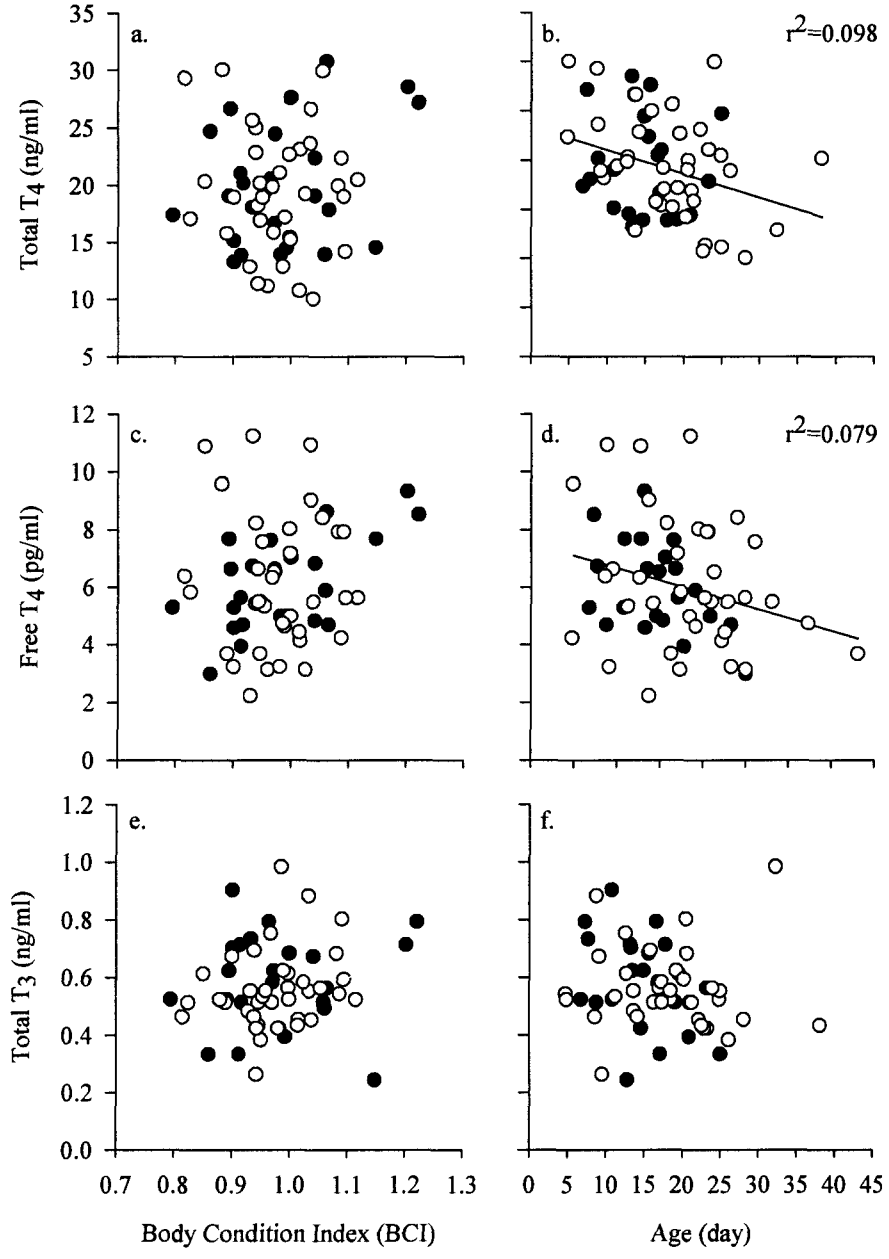

Fig 3.4. Circulating thyroid hormone concentrations from female (•) and male (○) SSL pups. (a, b) total T_4 (ng/ml); (c, d) free T_4 (pg/ml); (e, f) total T_3 (ng/ml) presented against the body condition index (BCI) or age. Female pups are represented by filled circles, males are by hollow circles. Only significant ($p \leq 0.05$) trend lines are presented.

3.5 Discussion

Changes in body condition and mass and associated changes in circulating cortisol, thyroid hormones and leptin have been well documented in several species of terrestrial mammals (Barboza et al. 2004; Buff et al. 2002; Caldeira et al. 2007; Concannon et al. 2001) and pinnipeds (Jeanniard du Dot et al. 2009; Ortiz et al. 2003). However, we found no relationship between BCI and any of the circulating concentrations of the hormones quantified in the present study on SSL. Further, body mass and a density index calculated as [body mass/ (standard length x axilary girth^2)] x 10^6 (Castellini et al. 1993; Rea 2002) were similarly not related to circulating concentrations of any hormones assessed in the present study (data not shown).

In the present study, pups were in a period of rapid growth as evident by the increase in body mass, standard length and axillary girth with age. Males between 5 and 38 days of age were larger than females in body mass, standard length and axillary girth. These findings have been previously reported for SSL pups (Brandon et al. 2005). Brandon et al. (2005) found that during the first six weeks following birth, male and female SSL pups grew at the same rate in body mass, standard length and axillary girth and suggested that males were larger than females at birth by approximately 15%. Estimated mass at birth (female 18.7 kg, male 22.4 kg) did not vary between rookeries while growth rates in body mass ranged from 0.23 kg/day to 0.48 kg/day with higher rates found in the Aleutian Islands (Brandon et al. 2005). Pups from the Chiswell Island rookery were not included in that study and Chiswell Island is geographically closer to rookeries with the lower growth rates. Yet, if we apply the growth rates calculated by Brandon et al. (2005) to SSL pups of known ages in the present study, then SSL pups from Chiswell Island rookery are estimated to be 4.1 and 17.9% (females) and 3.0 and 17.2% (males) larger than predicted by the lowest and maximum estimated growth rates, respectively. Further, pups in the present study on average weighed slightly more than predicated for females (1.96%) and males (2.42%) based on the BCI proposed by Trites and Jonker (2000). These findings are likely an indication that pups sampled in the present study are from a healthy population of SSL. Further, there were no abandoned or

starving pups observed on Chiswell Island at the time of blood collection (J. Maniscalco, personal communication).

While we detected a significant difference in size between male and female pups, suggesting either a difference in sex-related physiology or maternal investment during gestation as Brandon et al. (2005) proposed, only cortisol and aldosterone showed differences between male and female pups. Aldosterone is integral to the regulation of water and sodium balance in mammals. Within phocids, aldosterone concentrations significantly increased during extended periods of fasting in pups (Englehardt and Ferguson 1980; Nordoy et al. 1993; Ortiz et al. 2000). Unlike phocids, SSL pups have short periods of fasting associated with the maternal foraging trips, which tend to increase in length as the pup ages (Higgins et al. 1988; Maniscalco et al. 2006; Trites and Porter 2002). However, the large variation in aldosterone concentrations observed in the present study was not associated with age or BCI of the SSL pup and the difference between male and female pups, while statistically significant, was small and may not be biologically relevant. Cortisol is well known as a stress hormone and plays a role in gluconeogenesis and promoting the mobilization of fatty acids from peripheral adipose tissues. Cortisol concentrations in the present study increased as a result of the initial disturbance of the rookery and continually decreased as the time elapsed between the initial disturbance and collection of blood. Cortisol concentrations at the time of initial capture in free ranging juvenile SSL (Mellish et al. 2007) were comparable to pups in the present and previous studies (Myers et al. 2010). Similar to the present study, Myers et al. (2010) reported cortisol concentrations in female pups (155.2 ± 2.3 ng/ml) higher than male pups (141.5 ± 2.1 ng/ml). Higher baseline GC or metabolite concentrations have also been reported in adult female terrestrial mammals (Franceschini et al. 2007) and females exposed to an acute stressor exhibited higher peak cortisol concentrations than males (Guimont and Wynne-Edwards 2006). While the large sample size in the present study may have allowed for the detection of significant differences between male and female pups, the biological relevance of the small difference in cortisol and aldosterone concentrations between male and female pups is unclear.

Thyroid hormones including total and free T_4 and total T_3 are integral to maintaining thermoregulation, metabolism and the growth and development of neonatal and juvenile mammals. All forms of thyroid hormones quantified decreased when regressed against elapsed time since arrival on rookery, although the overall variation attributed to the elapsed time was low ($r^2<0.293$). The decrease in circulating total and free T_4 and total T_3 could be the result of the initial rookery disturbance and associated increased activity of pups leading to elevated concentrations of thyroid hormones followed by a period of decreasing concentrations as circulating hormones return to basal concentrations. Alternatively, adrenocorticotrophic hormone and glucocorticoids have been shown to alter the secretion of thyroid-stimulating hormone from the pituitary (Dussault 1974; Gartner et al. 1980; Wilber and Utiger 1969) and the conversation of T_4 to T_3 in tissues (Heyma and Larkins 1982; Kaplan and Utiger 1978) leading to reduced concentrations of thyroid hormones. Further, the elapsed time is by necessity later in the day and given the role of thyroid hormones in regulation of metabolism and thermoregulation, the decrease in thyroid hormones may not solely be related to the rookery disturbance but rather the result of increasing temperatures as the day progressed. Given the experimental design of the present study we are unable to determine the direct cause of the observed decrease in thyroid hormone concentrations over the elapsed time between arrival on rookery and sampling.

Mean thyroid hormone concentrations (total and free T_4, total T_3) were similar to those previously reported for SSL pups (Myers et al. 2006). However, while Myers et al. (2006) reported undetectable levels for some pups for all forms of thyroid hormones quantified, we did not have any SSL pups with undetectable levels for total and free T_4 or total T_3 but we were unable to quantify free T_3 (data not shown). Serum total T_3 was slightly lower in SSL pups but within the range reported in juvenile and sub-adult SSL (Jeanniard du Dot et al. 2009; Rosen and Kumagai 2008). SSL pups had total T_4 concentrations more than two times greater than baseline concentration during summer in juvenile and sub-adult SSL (Jeanniard du Dot et al. 2009; Rosen and Kumagai 2008) while free T_4 concentrations in SSL pups were half the circulating concentration found in

juvenile and sub-adult SSL (Jeanniard du Dot et al. 2009). Both total and free T_4 concentrations decreased with pup age, and while significant, the relationship was extremely weak (r^2=0.098) likely due to the short developmental period (5-38 days) covered in the present study. While male pups were larger than female pups at the time of sampling, no difference in circulating concentrations of any forms of thyroid hormones quantified was observed between male and female pups. These findings were not unexpected since Brandon et al. (2005) determined male and female pups have the same postnatal growth rate (body mass, standard length, and axillary girth) and concluded the difference in size between male and female pups was the result of differences in maternal investment during gestation.

Leptin is secreted by adipose tissue (Friedman and Halaas 1998; Kershaw and Flier 2004) and circulating concentrations have been correlated with fat stores and respond to changes in energy balance in terrestrial mammals (Friedman and Halaas 1998). We found higher leptin concentrations in pups physically restrained during blood collection compared to those under isoflurane. This finding was unexpected but driven by differences in fat stores or body mass as fluctuating leptin concentrations not associated with changes in fat or body mass have been previously reported in pinnipeds (Arnould et al. 2002; Mashburn and Atkinson 2008). In addition, the leptin concentrations in our study were near the lower detection limit of the kit (0.5 ng/ml) and similar to previous studies in phocids with similar methods (Hammond et al. 2005). Difficulty in quantifying leptin in SSL (Rosen and Kumagai 2008) and other pinnipeds (Arnould et al. 2002; Ortiz et al. 2003) has been previously reported with low concentrations or no correlation to body mass or lipid stores being reported (Arnould et al. 2002; Ortiz et al. 2001; Rosen and Kumagai 2008).

Circulating concentrations of cortisol, total and free T_4, and total T_3 decreased when regressed against the elapsed time between researchers' arrival on the rookery and removal of pup from the holding corral for blood collection. While the overall variation attributed to the rookery disturbance was low it may be of significance for future studies on free ranging pinnipeds. While we did not find BCI to be related to circulating hormone

concentrations future studies should include pups with a wider range of body conditions including abandoned or starving pups and ideally would employ a longitudinal sampling scheme to address some of the large individual variation in hormone concentrations observed in the present study. This study adds to the limited knowledge of the postnatal changes in free ranging SSL pups by providing concentration ranges of several hormones from an apparently healthy population. Further, given the unique life history traits of pinnipeds, these findings are also relevant for comparative endocrinology.

3.6 Acknowledgements

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Chapter 4:

Endocrine and immunological responses to adrenocorticotrophic hormone (ACTH) infusion in juvenile harbor seals (*Phoca vitulina*) during winter and spring

4.1 Abstract

Stressors activate the hypothalamic-pituitary-adrenal axis leading to secretion of adrenal hormones including glucocorticoids (GC) and aldosterone. GC have been shown to alter the hypothalamic-pituitary-thyroid axis and the immune system in mammals. Therefore, this study investigated the response of circulating concentrations of several hormones (cortisol, aldosterone, total and free thyroxine and total triiodothyronine) and immunological parameters (total and differential leukocyte counts and peripheral blood mononuclear cells (PBMC) proliferation to an acute stressor, exogenous adrenocorticotrophic hormone (ACTH), during two seasons in juvenile harbor seals. Cortisol concentrations peaked at 165 min in both winter (203.1 ± 84.7 ng/ml) and spring (205.3 ± 65.7 ng/ml) and were still significantly elevated 240 min after ACTH infusion. Aldosterone concentrations peaked at 90 min in both winter (359.3 ± 249.3 pg/ml) and spring (294.1 ± 83.7 pg/ml) and were still elevated 240 min after administration of ACTH in both seasons ($p \leq 0.011$). As in other mammalian species, juvenile harbor seals showed a significant increase in circulating total white blood cells driven primarily by the increase in neutrophil counts, which occurred simultaneously with a decrease in lymphocyte counts leading to an overall decrease in neutrophil to lymphocyte ratio. No clear trend was present in the *in vitro* PBMC proliferation following ACTH administration. There was not a seasonal difference for basal concentration of any hormone quantified or in their response to an acute stressor. Nor was there a seasonal difference in total and differential leukocyte counts or *in vitro* PBMC proliferation. These findings demonstrate that, similar to other mammalian species, an acute stress response in juvenile harbor seals results in an increase in cortisol and aldosterone concentrations, which is associated with altered immunological parameters.

4.2 Introduction

Stress is defined as a state of threatened homeostasis resulting from internal or external environmental change (Stratakis and Chrousos, 1995). The hypothalamic-pituitary-adrenal (HPA) axis is activated in response to a stressor leading to secretion of adrenal hormones including glucocorticoids (GC) and aldosterone (St. Aubin and Geraci, 1986; Thomson and Geraci, 1986; Gulland *et al.*, 1999; Ortiz and Worthy, 2000; Contreras *et al.*, 2004; Romano *et al.*, 2004). In addition to its role in the stress response, cortisol, the prominent GC in pinnipeds (DeRoos and Bern, 1961; Sangalang and Freeman, 1976), regulates gluconeogenesis promoting the mobilization of fatty acids from peripheral adipose tissues. In pinnipeds, cortisol has been suggested to play a role during molt (Riviere *et al.*, 1977; Ashwell-Erickson *et al.*, 1986; Boily, 1996), lactation (Engelhard *et al.*, 2002), fasting (Ortiz *et al.*, 2001a; Ortiz *et al.*, 2001b; Ortiz *et al.*, 2003a; Rosen and Kumagai, 2008) and potentially during diving (Zapol *et al.*, 1979). While aldosterone secretion is influenced by the HPA axis during a stress response, it is predominately under regulation by the renin-angiotensin system in pinnipeds (Malvin *et al.*, 1975; Ortiz *et al.*, 2000; Houser *et al.*, 2001; Ortiz *et al.*, 2003b; Ortiz *et al.*, 2006) with secretion stimulated by angiotensin II in order to maintain water and electrolyte balance in mammals. GC have also been shown to affect the hypothalamic-pituitary-thyroid (HPT) axis (Re *et al.*, 1976; Bianco *et al.*, 1987) and the immune system (Marketon and Glaser, 2008).

Thyroid hormones including total and free thyroxine (T_4) and total triiodothyronine (T_3) are essential for growth and development, metabolism and thermoregulation. Elevated GC concentrations following an acute stressor have been associated with changes in concentrations of T_4 and T_3. In free ranging beluga whales, T_4 and T_3 concentrations decreased during the 24 hrs following capture and handling (St. Aubin and Geraci, 1988). Similarly, total and free T_4 and total T_3 concentrations in free ranging Steller sea lion pups decreased following the initial rookery disturbance associated with research activities (Chapter 3). In both these studies on free ranging marine mammals basal concentrations of hormones were not known. Therefore, it was

not possible to determine if T_4 and T_3 continually decreased or initially increased followed by a subsequent decrease in response to a stressor. Studies on domesticated animals were able to establish basal concentrations of hormones prior to exposure to an acute stressor. In domestic camels transportation led to an initial increase in cortisol, T_4 and T_3 concentrations followed by a subsequent decrease resulting in these hormones returning to basal concentrations within 24 hr of the acute stressor (Saeb *et al.*, 2010).

Administrations of ACTH or cortisol are associated with characteristic changes in circulating populations of leukocytes and alterations in cell-mediated immune function in cattle and sheep (Roth and Kaeberle, 1981a; Roth and Kaeberle, 1981b). Circulating neutrophil counts increased rapidly with a concurrent decrease in lymphocyte and monocyte counts in association with elevated GC concentrations during restraint in rats (Dhabhar *et al.*, 1995; Dhabhar *et al.*, 1996). Further, suppression of immune function following an acute stressor included reduced *in vitro* proliferation of PBMC in piglets (Westly and Kelley, 1984) but not in lambs (Minton and Blecha, 1990; Coppinger *et al.*, 1991) or calves (Manak, 1986). The differences observed in the effect of elevated GC on immune parameters is dependent on the concentration of GC induced by the acute stressor, the immune parameter and the species being studied (Khansari *et al.*, 1990). Given the physiological influence of GC following exposure to an acute stressor, this study investigated the response of circulating concentrations of several hormones (cortisol, aldosterone, total and free T_4 and T_3) and immunological parameters (total and differential leukocyte count and PBMC proliferation) in harbor seals (*Phoca vitulina*) following administration of exogenous ACTH. I hypothesized that 1) exogenous ACTH would induce an acute stress response as demonstrated by an increase in cortisol concentrations; and 2) the acute stress response would be associated with altered immune parameters including an increase in total WBC and a decrease in *in vitro* PBMC proliferation.

The importance of body mass and composition in harbor seals is evident by the positive correlation of body mass and lipid stores with survival during the first year of independence (Muelbert *et al.*, 2003; Harding *et al.*, 2005). Further, mass of harbor seals

at birth and postnatal growth rates were influence by the mass of the adult female harbor seal, with heavier females giving birth to heavier pups with a faster growth rate (Ellis *et al.*, 2000; Bowen *et al.*, 2001). Harbor seals also display seasonal variation in body mass, lipid stores, and resting metabolic rate (Rosen and Renouf, 1997; Rosen and Renouf, 1998; Mellish *et al.*, 2007). Given the potential influence of body mass on the health and survival of harbor seals and the seasonality in body mass, we also assessed if body mass was related to an animal's ability to respond to an acute stressor. The objectives of the current study were to determine: (1) the effect of an acute stressor on the HPT axis and immune parameters; (2) if there is a difference in the physiological response to an acute stressor between seasons; and (3) if variation in body mass was associated with variations in the physiological response to an acute stressor as measured by circulating cortisol and aldosterone concentrations.

4.3 Materials and Methods

4.3.1 Animals and Blood Collection

Seven juvenile (1.5 – 3 years old), female harbor seals (*Phoca vitulina*) maintained at the Alaska SeaLife Center in Seward, Alaska (60° N latitude, 149° W longitude) participated in two ACTH administration trials during the winter (November/December 2006) and spring (May 2007). Seals were captured as pups from the declining population in Prince William Sound in 2004 (n=3) and 2005 (n=4). All seals were simultaneously participating in a long-term nutritional study (L. Polasek, personal communication) and were maintained on a mixed diet comprised of pollock (*Theragra chalcogramma*), capelin (*Mallotus villosus*), squid (*Loligo opalescens*) and either Pacific herring (*Clupea pallasii*) or Atlantic herring (*Clupea harengus*). Body mass (BM) was measured to the nearest tenth of a kilogram with a platform scale (Transcell Model TI-500-SL, Accurate Scales, Terre Haute, IN). The seals' mass ranged between 23.5 and 36.4 kg (mean 29.9 ± 1.3 kg) and they were considered healthy based on clinical and behavioral observations by veterinary and animal care staff.

During each trial seals were given a 5 μg/kg of Cortrosyn™ (Amphastar Pharmaceuticals, Inc. Rancho Cucamonga, CA) administered intramuscularly. This dose of Cortrosyn™, a synthetic ACTH, has been previously used in assessment of adrenal function in dogs (Watson *et al.*, 1998; Behrend *et al.*, 2006). Two hours prior to ACTH administration seals were given torbugesic (Fort Dodge; Fort Dodge, Iowa) and diazepam (Hospira Inc.; Lake Forest, Ill) prior to the placement of an indwelling 16 g x 15 cm long-term, guide wire style catheter (Mila International, Inc., Florence, KY, USA) in the extradural vein for serial blood sample collection. Seals were given two hours following catheter placement to recover prior to administration of ACTH. Blood was collected immediately prior to ACTH administration (0 min) and 60, 90, 165, 240 min after ACTH administration. Due to the limited number of seals participating in the study, no saline controls were used and blood collected prior to ACTH administration (time 0) was used for baseline values for all parameters in the present study. Bloods samples (< 17 ml) were drawn at each time point for a total blood volume for each seal ranging between 70 – 85 ml. Cell Preparation Tube (CPT) (CPT™ Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and Vacuette® blood collection tubes were kept upright and chilled until further processing in the lab (< 2 hrs). EDTA-treated blood tubes were collected for total and differential white blood cell (WBC) counts. CPT with sodium citrate was collected for isolation of PBMC. Serum was collected and stored at -80°C until hormone concentrations were quantified.

4.3.2 Hormone Radioimmunoassays

Commercially available radioimmunoassay (RIA) kits were used to quantify hormone concentrations in harbor seal serum. All samples were run in duplicate per manufacturer instructions with the exception that all volumes were halved. Solid-phase RIA kits (Siemens, Los Angeles, CA) were used for quantifying cortisol, aldosterone, total and free T_4 and total T_3 concentrations. Cortisol, total and free T_4 and total T_3 were previously validated for use with harbor seal serum in our laboratory (Oki and Atkinson, 2004) and aldosterone was validated in the present study. Pools of male and female

harbor seal serum were serially diluted to determine degree of parallelism to the standard curve. Recovery of added aldosterone (25–1200 pg/ml) for males (y=-21.591x + 173.63, r^2=0.996) and females (y=-23.63x + 282.29; r^2=0.998) was determined. Inter-assay and intra-assay coefficients of variation were < 10.0% for each hormone. Assay sensitivities were as follows: cortisol 2.0 ng/ml, aldosterone 11.0 pg/ml, total T_4 2.5 ng/ml, free T_4 0.1 pg/ml, and total T_3 0.07 ng/ml.

4.3.3. Total and Differential Leukocyte Counts

Total WBC counts were determined using the CBC-Diff Veterinary Hematological System (Heska ® Corporation, Loveland, CO). Blood smears were made from whole EDTA blood and stained with Wright-Giemsa (Dip Quick Stain, Jorgensen Laboratories, Loveland CO). WBC differentials, including neutrophils, lymphocytes, monocytes and eosinophils were counted manually and are reported as both percentages and absolute values of differentials.

4.3.4 PBMC Isolation and Proliferation

Blood collected into CPT tubes was processed per manufacturer instructions. Briefly, CPT tubes were centrifuged at 1500 x g for 20 min and the PBMC were re-suspended in freezing media comprised of RPMI 1640, 20% FBS (Hyclone, Thermo Fisher Scientific Inc., Logan, UT), 10% DMSO (Sigma Chemical Co., St. Louis, MO) and stored in liquid nitrogen until analysis. Cryopreserved PBMC were thawed and washed twice with PBS (without Ca^{2+}/Mg^{2+}) and re-suspended in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml), 1 mM sodium pyruvate, 100 µM nonessential amino acids, 25 mM hepes, and 0.05 mM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). All cell culture media and supplements, except for FBS, DMSO and 2-mercaptoethanol, were purchased from Gibco, Invitrogen Corp., Carlsbad, CA. Viability was >90% and assessed using the exclusion dye trypan blue (Sigma Chemical Co., St. Louis, MO).

PBMC were plated (2.0 x10^5 cells/well) in 96-well flat-bottom tissue-culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) and their proliferation assessed following exposure to either concanavalin A (ConA, Sigma Aldrich, St. Louis, MO) or lipopysaccharide (LPS055:B5, Sigma Aldrich, St. Louis, MO). PBMC were cultured at 37°C with 5% CO_2 in triplicate for each time point for all seven harbor seals in each of the following treatment groups; 1) unexposed control, 2) ConA suboptimal (0.1 µg/ml), 3) ConA optimal (1.0 µg/ml), 4) LPS suboptimal (1.0 µg/ml) and 5) LPS optimal (100 µg/ml). ConA is a plant-derived mitogen and preferentially stimulates T cells (Barta and Barta, 1993) while LPS from *Escherichia coli* is a B-cell activator (Wechsler-Reya and Monroe, 1996). The use of suboptimal and optimal mitogen concentrations have been used in previous pinniped studies (Levin *et al.*, 2005; Mori *et al.*, 2006; Keogh *et al.*, 2010). Mitogens were reconstituted in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). These conditions are similar to those previously used for pinnipeds (Levin *et al.*, 2005; Mori *et al.*, 2006; Keogh *et al.*, 2010).

All PBMC were assessed in two assays, one for each season, with PBMC collected from a captive adult Steller sea lion (*Eumetopias jubatus*) as an inter-assay control. PBMC were incubated for 48 hrs after which 20 µl of bromodioxyuridine (BrdU) was added for a final concentration of 10 µM BrdU and incubated for an additional 18 hrs for a total incubation period of 66 hrs. Proliferation was assessed with the Cell Proliferation Biotrack ELISA System version 2 (GE Healthcare, Piscataway, NJ) per manufacturer's instructions using Dynatech MRX Revelation microplate reader (Dynatech Laboratories, Inc, Chantilly, VA) at 450 nm with a reference wavelength of 650 nm. Data are presented as mean of the triplicates for optical density (OD) with standard error and as a stimulation index (SI; mean OD of cells exposed to mitogen/mean OD of cells in media only).

4.3.5 Statistical Analysis

Data were analyzed with Systat 10 (Systat Software, Inc, Point Richmond, CA). Data were analyzed with Systat 10 (Systat Software, Inc., Point Richmond, CA, USA). For the purposes of analyses ACTH trials conducted in November or December of 2006 were defined as winter while spring trials took place during May 2007. Repeated measures analysis of variance (ANOVA) was used to evaluate the influence of season for each parameter. Additionally, the area under the curve was calculated based on the trapezoid formula with reference to the baseline concentrations (AUC_I) for cortisol and aldosterone concentrations following administration of ACTH (Pruessner *et al.*, 2003; Fekedulegn *et al.*, 2007). Paired t-tests were used to determine differences between baseline values at 0 min and the AUC_I between seasons. Pearson correlations were used to assess the association of body mass with basal hormone concentrations, total and differential WBC counts and *in vitro* proliferation of PBMC within each season. Results were considered statistically significant at $p \leq 0.050$.

4.4 Results

4.4.1 Changes in Hormone Concentrations Following ACTH Administration

Basal (0 min) serum cortisol concentrations did not differ (Table 4.1, p=0.461) between winter (28.5 ± 10.3 ng/ml) and spring (35.8 ± 21.6 ng/ml). Nor did basal aldosterone concentrations differ (p=0.472) between winter (18.2 ± 8.4 pg/ml) and spring (38.1 ± 29.6 pg/ml). In both seasons cortisol ($p \leq 0.001$) and aldosterone ($p \leq 0.001$) significantly increased in response to ACTH administration (Figure 4.1). Circulating concentrations of cortisol (winter: $p<0.001$; spring: p=0.001) and aldosterone (winter: p=0.003; spring: $p<0.001$) were significantly increased 60 min after administration of ACTH compared to basal concentrations in all seals and in both seasons (Figure 4.1). Cortisol concentrations peaked at 165 min in both winter (203.1 ± 84.7 ng/ml) and spring (205.3 ± 65.7 ng/ml) and were still significantly elevated 240 min after ACTH administration in both seasons ($p \leq 0.001$). Aldosterone concentrations peaked at 90 min in both winter (359.3 ± 249.3 pg/ml) and spring (294.1 ± 83.7 pg/ml) and were still elevated

240 min after administration of ACTH in both seasons ($p \leq 0.011$). There was no significant difference between seasons in cortisol ($p=0.872$) or aldosterone ($p=0.732$) concentrations in response to ACTH administration (Figure 4.1). Similarly, there was no difference between AUC_I between seasons for cortisol ($p=0.287$) or aldosterone ($p=0.326$) (Figure 4.1). In both seasons aldosterone and cortisol concentrations were positively correlated ($p \leq 0.003$).

There was a large amount of individual variation observed in the response of thyroid hormones to ACTH administration (Figure 4.2). Circulating concentrations of total T_4 ($p=0.032$) and total T_3 ($p=0.011$) but not free T_4 ($p=0.522$) significantly changed in response to ACTH stimulation (Figure 4.2). There was not a seasonal difference in the response to ACTH stimulation for total T_4 ($p=0.116$), free T_4 ($p=0.366$), nor total T_3 ($p=0.861$).

There was no difference between winter and spring for body mass (winter 29.4 ± 5.3 kg; spring 30.5 ± 4.6 kg; $p=0.184$). Basal cortisol concentrations were correlated to body mass in winter ($p=0.039$) but not in the spring ($p=0.311$). Further, there was no relationship between mass and cortisol concentrations at any time following ACTH administration in winter ($p<0.087$). In spring, response in cortisol to ACTH administration was related to body mass ($p=0.046$). Basal aldosterone concentrations were not correlated to body mass in either season ($p>0.154$). Further, there was no relationship between body mass and aldosterone concentrations at any time following ACTH administration in either season ($p<0.112$).

Table 4.1. Basal (time 0) mean (±SD) and range of cortisol, aldosterone, total and differential blood cell counts and PBMC proliferation by season from captive juvenile harbor seals (n=7).

	Winter		Spring	
Parameter	Mean ± SD	Range	Mean ± SD	Range
Cortisol (ng/ml)	28.5 ± 10.3	13.2 – 46.7	35.8 ± 21.6	18.0 – 75.4
Aldosterone (pg/ml)	18.2 ± 8.4	11.1 – 35.1	30.8 ± 29.6	4.9 – 72.0
Total thyroxine (ng/ml)	26.8 ± 5.2	18.6 – 33.5	21.9 ± 5.0	13.9 – 28.3
Free thyroxine (pg/ml)	6.4 ± 2.3	3.6 – 9.9	4.9 ± 1.4	3.2 – 7.3
Total triiodothyronine (ng/ml)	0.7 ± 0.2	0.5 – 0.1	0.74 ± 0.1	0.6 – 0.9
WBC (10^3/μl)	8.6 ± 1.5	6.7 – 11.0	7.9 ± 1.5	6.3 – 10.0
Neutrophil (10^3/μl)	5.4 ± 0.7	4.7 – 6.5	5.0 ± 1.3	2.8 – 7.1
(% of WBC)	66.0 ± 8.3	56 – 72	63.1 ± 7.5	54 – 72
Lymphocyte (10^3/μl)	1.9 ± 0.7	1.1 – 2.9	2.1 ± 0.7	1.6 – 3.4
(% of WBC)	22.4 ± 4.7	17 – 29	26.3 ± 5.2	7 – 20
Monocytes (10^3/μl)	0.7 ± 0.3	0.3 – 1.1	0.5 ± 0.3	0.1 – 0.9
(% of WBC)	8.9 ± 3.1	4 – 14	5.7 ± 2.6	1 – 9
Eosinophil (10^3/μl)	0.2 ± 0.2	0.0 – 0.5	0.1 ± 0.1	0.1 – 0.3
(% of WBC)	2.0 ± 2.3	0 – 5	1.7 ± 0.7	1 – 3
N:L ratio	3.1 ± 1.0	1.9 – 4.3	2.6 ± 0.9	1.6 – 4.0
Control (OD)	0.29 ± 0.02	0.25 – 0.33	0.30 ± 0.05	0.23 – 0.36
ConA 0.1 μg/ml (OD)	0.29 ± 0.01	0.26 – 0.30	0.31 ± 0.05	0.29 – 0.39
ConA 0.1 μg/ml (SI)	1.01 ± 0.07	0.88 – 1.10	1.06 ± 0.08	0.91 – 1.19
ConA 1.0 μg/ml (OD)	1.57 ± 0.58	0.29 – 1.95	2.00 ± 0.30	1.69 – 2.63
ConA 1.0 μg/ml (SI)	5.55 ± 2.18	0.95 – 7.50	6.87 ± 1.12	5.22 – 8.81
LPS 055:B5 1.0 μg/ml (OD)	0.37 ± 0.09	0.29 – 0.53	0.58 ± 0.19	0.41 – 0.83
LPS 055:B5 1.0 μg/ml (SI)	1.27 ± 0.23	0.94 – 0.63	1.51 ± 0.39	0.94 – 1.63
LPS 055:B5 100 μg/ml (OD)	0.68 ± 0.28	0.28 – 1.01	0.88 ± 0.35	0.53 – 1.60
LPS 055:B5 100 μg/ml (SI)	2.38 ± 1.04	0.91 – 3.97	2.91 ± 0.77	2.10 – 4.50

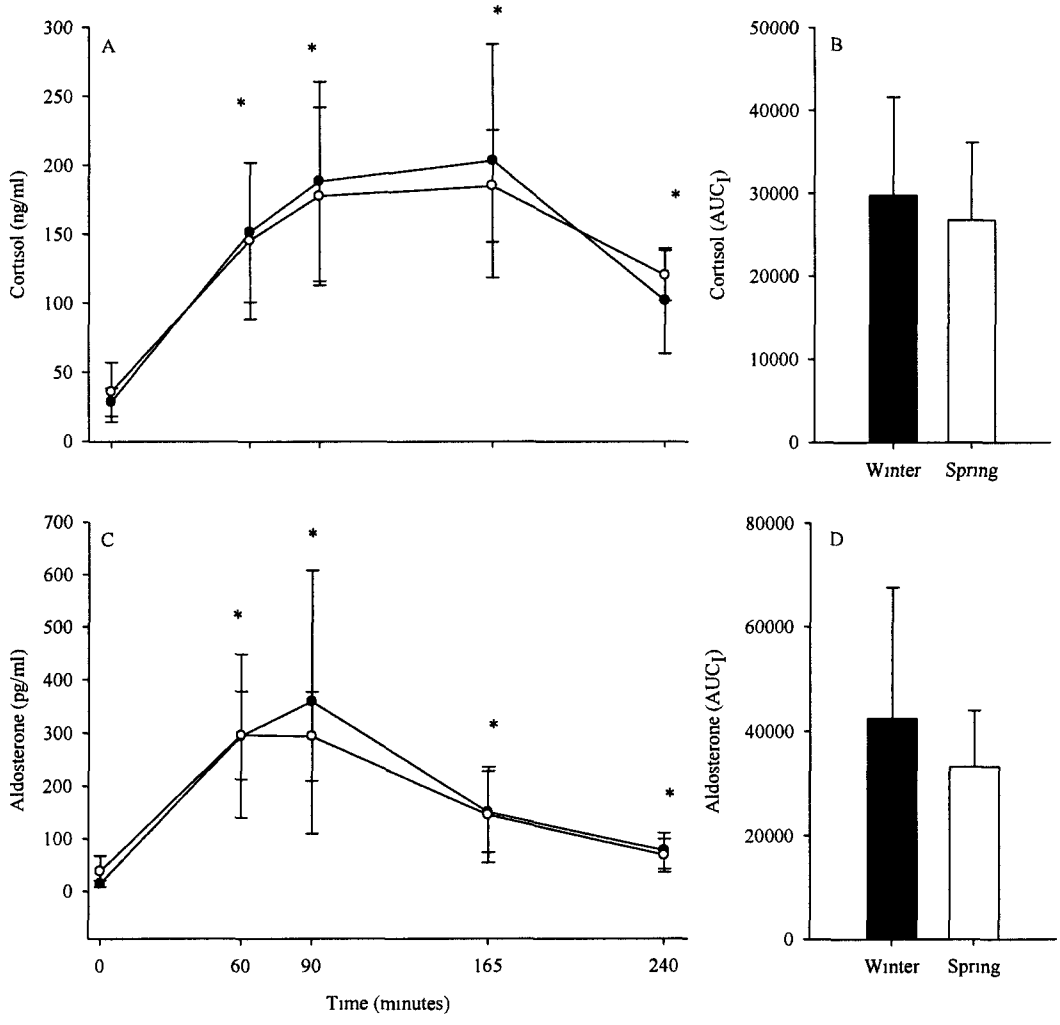

Figure 4.1. Mean (± SD) serum cortisol (A) and aldosterone (C) concentrations over time following ACTH injection during the winter (●) and spring (○) for seven juvenile harbor seals (n=7). Area under the curve (AUC_I) following ACTH injection for cortisol (B) and aldosterone (D) during the winter and spring experiments. * denotes significant ($p < 0.05$) difference from time 0.

4.4.2 Alterations in Total and Differential Leukocyte Counts

There was no difference in response to ACTH administration between seasons in circulating total WBC count (Figure 4.3, $p=0.241$). Nor was there a difference in neutrophils, monocytes or eosinophils whether expressed as percentage or absolute number ($p>0.062$), or neutrophil: lymphocyte (N: L) ratio ($p<0.142$). There was a significant effect of season on circulating lymphocytes in response to ACTH administration as a percentage ($p=0.020$) with winter having lower percent of lymphocytes compared to spring. This relationship was not found when lymphocytes were reported as an absolute count ($p=0.537$). Total WBC count ($p<0.001$), neutrophils (percentage, $p< 0.001$; absolute count, $p<0.001$), and lymphocytes (percentage, $p\leq 0.001$; absolute count, $p< 0.001$) changed following ACTH administration (Figure 4.3). However, no change was observed in circulating monocytes (percentage, $p=0.229$; absolute count, $p=0.386$). Specifically, total WBC, neutrophil counts and N: L ratios were significantly elevated 240 min after ACTH administration ($p<0.021$) while lymphocyte count decreased ($p< 0.007$). Further, there was no relationship between total or differential WBC count and between body mass for either season ($p> 0.141$).

4.4.3 PBMC in vitro Proliferation

There was no difference in response to ACTH administration between seasons in the spontaneous (unexposed) or stimulated (ConA or LPS) PBMC proliferation ($p> 0.089$). Nor did spontaneous ($p=0.830$) or suboptimal ConA (OD, $p=0.243$; SI, $p=0.752$) PBMC proliferation change following ACTH administration. There was no difference in PBMC proliferation between seasons when stimulated by optimal ConA but proliferation did significantly change following ACTH administration (OD, $p< 0.001$; SI, $p< 0.001$); however, while there was a clear pattern, there was not an overall trend present (Figure 4.4). PBMC-proliferation did significantly change following ACTH administration when stimulated by suboptimal (OD, $p=0.006$; SI, $p=0.036$) and optimal LPS (OD $p=0.001$; SI $p=0.005$). Similar to the response in stimulation by optimal ConA, proliferation following *in vitro* exposure to LPS (optimal and suboptimal) showed a clear pattern but with no obvious trend. *In vitro* PBMC proliferation was only positively correlated with body

mass following stimulation with optimal LPS at time 0 in the winter when reported as an OD (p=0.046) but not SI (p=0.120).

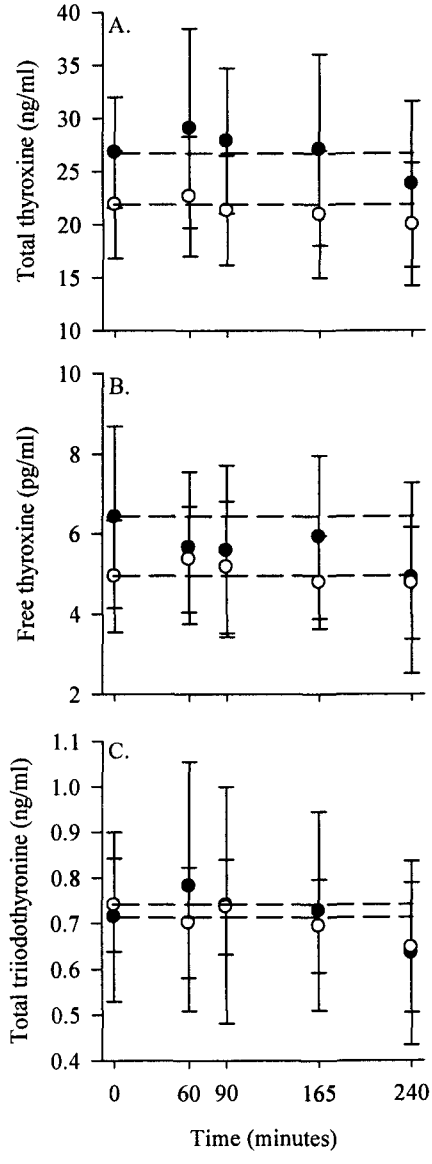

Figure 4.2. Mean (± SD) serum concentration for total (A) and free (B) thyroxine and total triiodothyronine (C) following ACTH injection during the winter (●) and spring (○) for seven juvenile harbor seals (n=7). Dashed lines denote baseline concentrations.

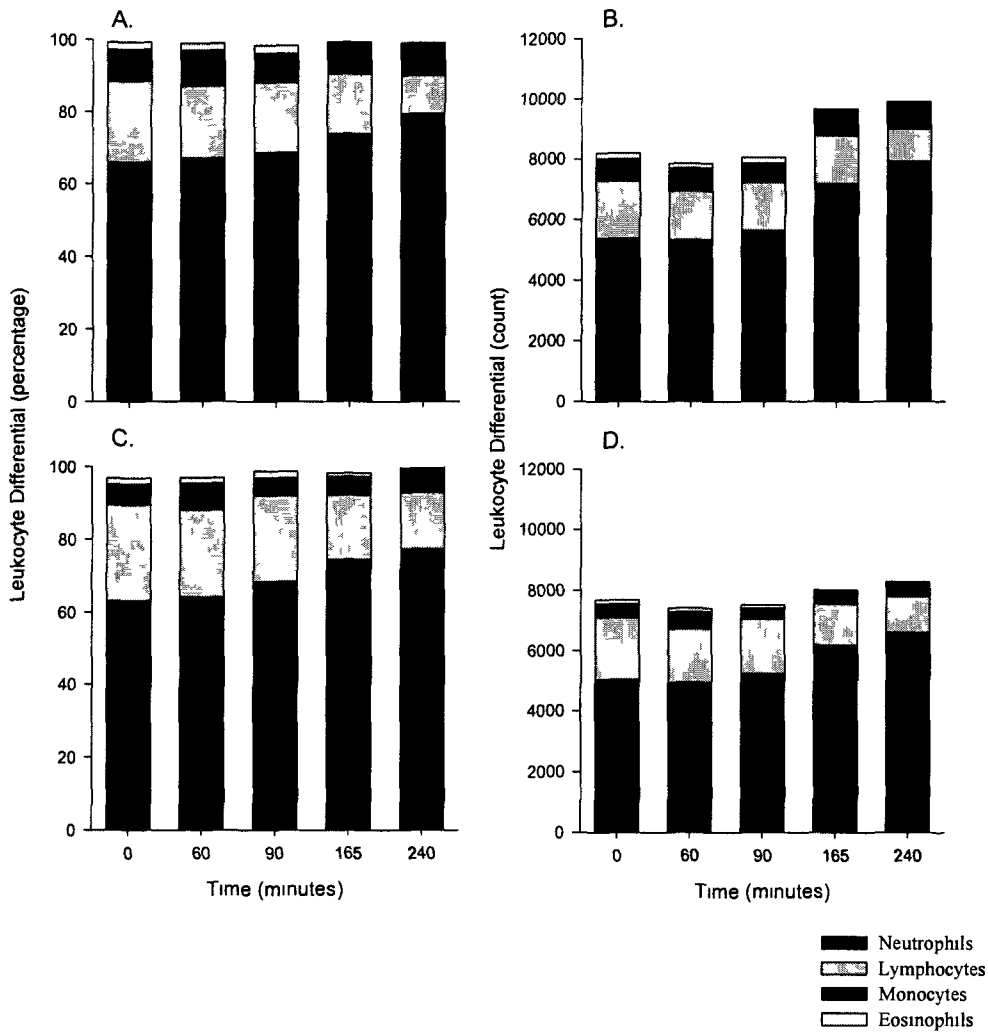

Figure 4.3 Mean differential leukocyte counts for all seven juvenile harbor seals following an acute stressor (exogenous ACTH) during the winter with results reported as percentage (A) and absolute count (B) and during spring with results reported as percentage (C) and absolute count (D).

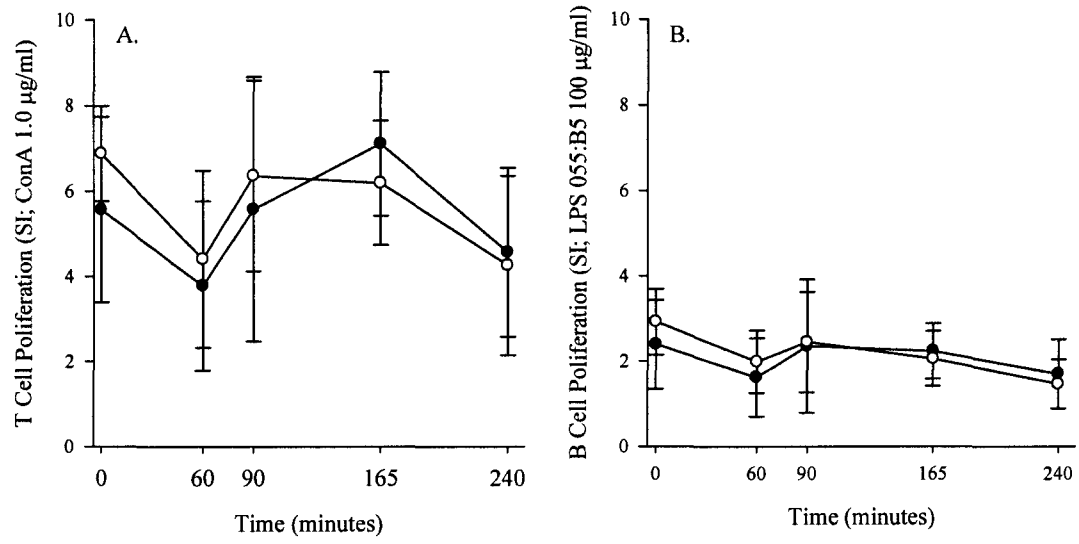

Figure 4.4 PBMC proliferation responses from seven captive juvenile harbor seals exposed to (A) 1.0 µg/ml ConA and (B) 100 µg/ml LPS following an acute stressor during winter (●) and spring (○). Results presented as mean stimulation index (SI) ± SD.

4.5 Discussion

4.5.1 Hormone Concentrations Induced by ACTH

An acute stress response was induced in all seals as evident by the changes in cortisol concentrations following administration of 5 µg/kg of Cortrosyn™. Gulland *et al.*, (1999) reported cortisol concentrations prior to ACTH administration higher in harbor seal pups (2-4 months old) during rehabilitation than basal cortisol concentrations in the present study. Similarly, basal cortisol concentrations previously reported for wild and captive harbor seals (Gardiner and Hall, 1997) were higher than concentrations in present study. Peak cortisol concentrations following exogenous ACTH gel (Gulland *et al.*, 1999) resulted in similar peak concentrations as in the present study; however, peak cortisol were observed later (165 min) following ACTH administration in the present study than in the previous study (90 min) (Gulland *et al.*, 1999). These results may be explained by differences in the methodologies employed in the studies. In the present study, an indwelling catheter was used for serial blood collection, following the

placement of which seals were allowed to rest for 2 hrs prior to administration of ACTH. This experimental set up eliminated the need to physically restrain the seals during blood collection and allowed seals to recover from the increase in cortisol concentrations measured at the time of the catheter placement (data not shown). During the 240 min following ACTH administration in the present study, seals remained calm and most slept for large periods of time. It is important to note that the cortisol concentrations observed in the present study including the peak observed at 165 min following ACTH administration were lower than reported concentrations of cortisol in wild harbor seals (Gardiner and Hall, 1997; Gulland *et al.*, 1999). This finding was not unexpected given the differences between harbor seal populations and since different stressors result in different behavioral and physiological responses including cortisol concentrations. The increase in cortisol concentrations differed based on capture methods used in bears (Cattet *et al.*, 2003), white-tailed deer (Delgiudice *et al.*, 1990; Denicola and Swihart, 1997), bottlenose dolphins (Thomson and Geraci, 1986) and elephant seals (Engelhard *et al.*, 2002).

While cortisol concentrations have been frequently reported for harbor seals in relation to molt (Riviere *et al.*, 1977; Ashwell-Erickson *et al.*, 1986), breeding (Gardiner and Hall, 1997), seasonal and diurnal patterns (Gardiner and Hall, 1997; Oki and Atkinson, 2004) there is comparatively limited information available for aldosterone concentrations in harbor seals (St. Aubin and Geraci, 1986; Gulland *et al.*, 1999). The present study showed a significant increase in aldosterone concentration, which was correlated with cortisol concentrations following ACTH administration, demonstrating that secretion of aldosterone is also under the HPA axis control as has been previously demonstrated in phocids (St. Aubin and Geraci, 1986; Gulland *et al.*, 1999). Basal aldosterone concentrations or concentrations in response to ACTH administration were not related to season. Similar to cortisol, basal aldosterone concentrations in the present study were lower during both seasons than previous reported initial aldosterone concentrations in harbor seals pups during rehabilitation or wild harbor seals (Gulland *et al.*, 1999). Gulland *at al.*, (1999) reported a peak in aldosterone concentrations 90 min

following ACTH administration, similar to the present study; however, harbor seal pups given an ACTH administration during rehabilitation had more than a 4 times greater aldosterone concentrations compared to the 90 min peak in the present study. Given that aldosterone secretion is under the control of HPA as well as renin-angiotensin system, the higher aldosterone concentrations previously reported for harbor seals (Gulland *et al.*, 1999) may be due to disease and physiological differences between study populations and methodologies such as different ACTH and restraint during blood collections.

Basal cortisol concentrations were correlated to body mass in winter but not spring, which may indicate that body mass is more critical during the winter. Similar to the lack of seasonal differences in body mass in the present study we found no seasonal difference in cortisol or aldosterone concentrations in response to exogenous ACTH. While these findings may be, at least in part, due to the seals being immature, captive seals have been reported to lack seasonal variation in cortisol concentrations observed in wild seals (Gardiner and Hall, 1997). Gardiner and Hall (1997) found circulating cortisol concentrations in mature captive females did not show the seasonal variation observed in mature wild female and male harbor seals, which was attributed to the consistent environment and availability of food in the captive environment.

4.5.2 Alterations in Total and Differential Leukocyte Counts

The basal WBC count in the present study was similar to previously published results for captive harbor seals (McConnell and Vaughan, 1983). Compared to wild adult harbor seals (Ross *et al.*, 1993; Morgan *et al.*, 1998) baseline WBC, neutrophil, and eosinophil counts were lower while lymphocyte and monocytes counts were higher in the present study. Differences in total and differential WBC counts may be indicative of population difference. However, differences in total and differential white blood cell counts between immature and mature mammals have been reported across taxa including carnivore (Nunn *et al.*, 2009). Therefore, the differences in total and differential leukocyte counts observed in the present study may be attributed to age, since Nielsen (1995) found neutrophils (%) increased while lymphocytes (%) decreased with age. The

lower eosinophil counts observed in the present study as in other captive seal populations are likely due to the expected lower occurrence of parasites in captive compared to wild animals. In the present study total and differential WBC counts were not related to body mass. Across mammalian species the number of circulating total white blood cell counts increased with body mass in adults (Nunn *et al.*, 2003; Nunn *et al.*, 2009). However, the present study did not find body mass to be related to total WBC count.

The immune system is not independent but rather there is a well-established link between the immune and endocrine systems (Haddad *et al.*, 2002; Weber, 2003). Capture and handling methods routinely used during blood collection in wild mammals can result in elevated cortisol concentrations (Engelhard *et al.*, 2002; Romero *et al.*, 2008; Keogh *et al.*, 2010), which may alter hematological parameters (Castellini *et al.*, 1996; Cattet *et al.*, 2003). Circulating neutrophil counts increase rapidly with a concurrent decrease in lymphocyte and monocyte counts during an acute stress response in association with elevated GC concentrations in terrestrial mammals (Dhabhar *et al.*, 1995; Dhabhar *et al.*, 1996; Cattet *et al.*, 2003). Similarly, an increase in total WBC, neutrophil and neutrophil to lymphocyte ratio was associated with increases in cortisol concentration following ACTH administration in the present study. The observed increase in total WBC and neutrophil to lymphocyte ratio was driven predominately by the increase in neutrophils. Juvenile harbor seals response to an acute stressor in the present study was typically mammalian exhibiting a stress leukogram.

4.5.3 PBMC in vitro Proliferation

PBMC including lymphocytes play an important role in cell-mediated immunity. The immunosuppressive effect of elevated GC concentration associated with stressors has been well established in mammals (Keller *et al.*, 1981; Deguchi and Akuzawa, 1998; Bilandzic *et al.*, 2006). Elevated cortisol concentrations have been shown to suppress PBMC proliferation in piglets (Westly and Kelley, 1984) while having no influence on lambs (Minton and Blecha, 1990) or calves (Manak, 1986). The effect of elevated GC on immune parameters is dependent on several variables including the concentration of GC

induced by an acute stressor, the immune parameter or function, and the species being studied (Bilandzic *et al.*, 2006). Previous studies on the acute stress response in seals (St. Aubin and Geraci, 1986; Gulland *et al.*, 1999) have not assessed the impact of an acute stressor on total and differential leukocyte count or *in vitro* PBMC proliferation. Therefore, we assessed the effect of an acute stressor (exogenous ACTH) on *in vitro* PBMC proliferation in addition to total and differential leukocyte counts. While we did not quantify the number of circulating T and B cells, the use of two mitogens that preferentially stimulate either T cells (Barta and Barta, 1993) or B cells (Wechsler-Reya and Monroe, 1996) allowed the assessment of both cell populations. All harbor seals in the present study responded to *in vitro* stimulation with ConA and LPS 055:B5 indicating peripheral T and B cells are capable of responding to an antigenic challenge as previously reported for harbor seals (Ross *et al.*, 1993; Swart *et al.*, 1993; Levin *et al.*, 2005). As with previous studies which assessed PBMC proliferation in harbor seals, T cells proliferated more so than B cells when exposed to *in vitro* mitogens at optimal and suboptimal concentrations (Swart *et al.*, 1993; Levin *et al.*, 2005). The lower B cell proliferation in these studies may be the result of the low number of B cells in the peripheral blood as observed in other mammals (Byrne *et al.*, 2000; Faldyna *et al.*, 2001). However, the ratio of T and B cells in harbor seals will need to be confirmed in future studies. T cell proliferation in the present study, regardless of time after ACTH administration was higher than reported for wild lactating female and harbor seal pups (Ross *et al.*, 1993). The SI for B cell proliferation were within the range previously reported for harbor seals (Levin *et al.*, 2005). Levin *et al.*, (2005) reported a negative relationship between B cell proliferation (SI) and body mass in harbor seal pups which was not observed in the present study. However, B cells proliferation in the present study was related to body mass in the winter for optimal LPS when reported as OD but this relationship was not maintained when proliferation was reported as SI.

4.5.4 Body Mass

Body mass has been shown to be important in the health and survival of harbor seals (Bowen *et al.*, 2001; Muelbert *et al.*, 2003; Harding *et al.*, 2005). Body mass is influenced by an individual's disease and nutritional state; therefore, we assessed the influence of body mass on the seal's response to an acute stressor. Harbor seals have been observed to first give birth between 4 and 6 years of age (Markussen *et al.*, 1989), therefore the female harbor seals (1.5 – 3 years old) in the present study were likely immature. As expected, seals in the present study had lower body mass compared to mature seals (Ellis *et al.*, 2000; Bowen *et al.*, 2001; Mellish *et al.*, 2007). The juvenile harbor seals in the present study showed no seasonal variation in total body mass and had a lower body mass in both seasons than total body mass reported for wild yearling (body mass, 33.3 ± 1.3 kg) and juvenile (44-61 kg) harbor seals (Swart *et al.*, 1994; Muelbert *et al.*, 2003). Mature harbor seals have been reported to display seasonal variation in body mass, lipid stores (Rosen and Renouf, 1997; Mellish *et al.*, 2007), energy intake (Rosen and Renouf, 1998), and resting metabolic rate (Rosen and Renouf, 1998). However, seasonal variation was not found in body mass, energy intake or blubber stores in previous studies on juvenile harbor seals (Pitcher, 1986; Markussen and Ryg, 1990). Therefore, it may be that the lack of seasonal variation in body mass in the present study is due, at least in part, to the seals being immature.

4.6 Conclusions

Juvenile harbor seals did not show seasonal variation in any parameter measured, nor did body mass affect the response to an acute stressor in the present study. However, basal cortisol concentrations were related to body mass in the winter, which is suggestive that body mass in the winter may be important for pinnipeds as suggested by Rosen and Kumagai (2008). Circulating concentrations of cortisol and aldosterone significantly increased in response to an acute stressor (exogenous ACTH), which resulted in a stress leukogram at 240 min after application of the stressor. As in other mammalian species, juvenile harbor seals showed a significant increase in total WBC count driven primarily

by the increase in neutrophil counts, which occurred simultaneously with a decrease in lymphocyte counts leading to an overall decrease in N: L ratio. No clear trend was apparent in the *in vitro* proliferation of T or B cells following ACTH administration.

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Chapter 5:

General Conclusions

This study assessed the "health" of individual animals by quantifying circulating concentrations of hormones associated with nutritional status and fat mass (leptin), lipid and water metabolism (cortisol and aldosterone), and growth and metabolism (thyroxine and triiodothyronine) as well as circulating total and differential leukocyte counts and *in vitro* proliferation of PBMC.

5.1.1 Hormones, Body Mass, and Age

There is strong evidence supporting the importance of body mass in reproductive success and survival of pups in phocids and otariids (Baker and Fowler, 1992; Arnbom *et al.*, 1994; Kjellqwist *et al.*, 1995; Ellis *et al.*, 2000; Bowen *et al.*, 2001; Harding *et al.*, 2005). Further, changes in body condition or mass in juvenile and adult pinnipeds have been associated with changes in circulating cortisol, thyroid hormones and leptin in several species of terrestrial mammals (Concannon *et al.*, 2001; Buff *et al.*, 2002; Barboza *et al.*, 2004; Caldeira *et al.*, 2007) and pinnipeds (Ortiz *et al.*, 2003; Jeanniard du Dot *et al.*, 2009). However, in the present studies body condition index was not related to any hormones quantified in SSL pups (Chapter 3). Further, age of SSL pups during the early postnatal period, while significant was only weakly related to total and free T_4 concentrations (r^2=0.098). When compared to juvenile and sub-adult SSL (Rosen and Kumagai, 2008; Jeanniard du Dot *et al.*, 2009), SSL pups (Chapter 3) had two times greater total T_4 concentrations while free T_4 concentrations were half the circulating concentration found in juvenile and sub-adult SSL (Jeanniard du Dot *et al.*, 2009). Therefore, the weak relationship between age and thyroid hormone concentrations in SSL pups is likely due to the short developmental period (5-38 days) covered in the present study (Chapter 3), suggesting that a longitudinal study which covers a longer

development period is likely needed to fully explore the influence of age on circulating thyroid hormone concentrations.

The relationship of leptin concentrations and body mass in pinnipeds remains unclear. Previous studies have reported difficulty in quantifying leptin in SSL (Rosen and Kumagai, 2008) and other pinnipeds (Arnould *et al.*, 2002; Ortiz *et al.*, 2003) have been previously reported with low concentrations or no correlation to body mass or lipid stores (Ortiz *et al.*, 2001; Arnould *et al.*, 2002; Rosen and Kumagai, 2008). The present study (Chapter 3) does not clarify the role of leptin in pinnipeds. Leptin concentrations in our study were near the lower detection limit of the kit (0.5 ng/ml) similar to previous studies in phocids with similar methods (Hammond *et al.*, 2005).

BCI or body mass and nutritional status have showed seasonality in phocids (Ryg *et al.*, 1990; Beck *et al.*, 1993; Renouf *et al.*, 1993; Nilssen *et al.*, 2001; Chabot and Stenson, 2002) and otariids (Kumagai *et al.*, 2006; Mellish *et al.*, 2007; Rea *et al.*, 2007; Williams *et al.*, 2007; Jeanniard du Dot *et al.*, 2008). Juvenile HS did not show seasonal variation in cortisol or aldosterone, nor did body mass or composition affect the response to an acute stressor in the present study (Chapter 4). Sexually mature HS have been reported to display seasonal variation in body mass, lipid stores (Rosen and Renouf, 1997; Mellish *et al.*, 2007), energy intake (Rosen and Renouf, 1998), and resting metabolic rate (Rosen and Renouf, 1998). However, seasonal variation was not found in body mass, energy intake or blubber stores in previous studies of juvenile HS (Pitcher, 1986; Markussen and Ryg, 1990). Therefore, the lack of seasonality may be due to HS in the present study being immature (Chapter 4). Given these findings exogenous ACTH administration should be applied to sexually mature HS to determine if seasonality is present in the response to an acute stressor which was not present in the juvenile HS (Chapter 4).

Overall, the findings of this study indicate that mass and body condition did not influence endocrine or immune profiles of juvenile HS and SSL pups. However, caution should be taken given the short developmental period (SSL pups) and low variation in body condition and mass (HS and SSL pups) covered in the present study. When the

findings of the present study are compared to published studies on SSL and HS, there does appear to be an effect of age, at least as classified as mature vs. immature animals in thyroid hormones and total and differential leukocyte counts.

5.1.2 Total and Differential Leukocyte Counts

Across mammalian species the number of circulating total white blood cell counts has been shown to increase with body mass in adults (Nunn *et al.*, 2003; Nunn *et al.*, 2009). However, total and differential WBC counts were not related to body mass or composition in juvenile HS (Chapter 4). Compared to wild adult HS (Ross *et al.*, 1993; Morgan *et al.*, 1998) baseline WBC, neutrophil, and eosinophil counts were lower while lymphocyte and monocytes counts were higher in the present study (Chapter 4). Differences in total and differential white blood cell counts between immature and mature mammals have been reported across taxa including carnivore (Nunn *et al.*, 2009). Therefore, differences in total and differential leukocyte counts observed in the present study (Chapter 4) may be related to age of the HS. Nielsen (1995) found that total WBC count decreased in association of an increase in neutrophils (%) with a concurrent decrease in lymphocytes (%) with age (0.75 - 17.5 years of age) of HS. Therefore, the decrease in WBC was driven primarily by a reduced number of circulating lymphocytes. When HS pups were monitored during the early postnatal period, pups showed an initial increase in WBC count, which was maintained until at least weaning (Ross *et al.*, 1993; Ross *et al.*, 1994). The influence of age on total and differential leukocytes in HS differs from the findings for SSL pups in the present study (Chapter 2). In SSL pups, total WBC counts showed a decrease between 5 and 38 days of age. Unlike HS, the decrease in total WBC count was primarily driven by a decrease in neutrophils while no change was observed in lymphocyte counts. These findings along with previous studies on older pups (6 to 10 months of age; Mellish *et al.*, 2006) and juveniles (1 to 3 years; Mellish *et al.*, 2006) indicate that young SSL pups have a higher WBC count at birth followed by a postnatal decrease remaining below the levels observed in juveniles. Previous studies on SSL have not reported differential leukocyte counts and therefore further comparisons in

changes of neutrophil and lymphocyte populations are not possible. However, it is likely that the reported change in total WBC counts in SSL is driven predominately by changes in circulating neutrohpil counts. These findings are suggestive that SSL and HS pups show different patterns of changes in the circulating populations of leukocytes during the early postnatal period. Previous studies on HS pups and the current study on SSL pups (Chapter 2) have explored postnatal changes in PBMC function by *in vitro* mitogen stimulation. However, to date no studies on either HS or SSL have explored changes to the innate immune response. Given the changes associated with age in circulating numbers of neutrophils observed in both HS and SSL, future studies should explore immunological functions of neutrophils, such as phagocytic and respiratory burst activity and the effect of age.

Studies focusing on neutrophils may provide informative not only in regards to functional changes associated with age or maturity but also in regards to the effects of an acute stressor on neutrophils. Elevated cortisol concentrations have been associated with capture and handling methods routinely used during blood collection in wild mammals (Engelhard *et al.*, 2002; Romero *et al.*, 2008) including in the present study (Chapter 2, Chapter 3). GC have been shown to negatively affect some components of the immune system (Marketon and Glaser, 2008) with administration of ACTH or cortisol producing characteristic changes in circulating populations of leukocytes (Roth and Kaeberle, 1981a; Roth and Kaeberle, 1981b). During an acute stress response circulating neutrophil counts increase rapidly with a concurrent decrease in lymphocyte and monocytes (Dhabhar *et al.*, 1995; Dhabhar *et al.*, 1996; Cattet *et al.*, 2003). Similarly an increase in total WBC, neutrophil and neutrophil to lymphocyte ratio was associated with an increase in cortisol concentration following ACTH administration in juvenile HS (Chapter 4). These findings demonstrate that juvenile HS response to an acute stressor is typically mammalian exhibiting a stress leukogram. However, the number of circulating lymphoyctes and the *in vitro* proliferation of PBMC did not change in association with rookery disturbance and associated handling in SSL pups (Chapter 2) or following administration of ACTH in juvenile HS (Chapter 4). The innate immune response of

neutrophils was not explored and given the increase in circulating neutrophils associated with an acute stressor in juvenile HS (Chapter 4), future studies should include assays to assess the function of neutrophils including phagocytic and respiratory burst activity.

5.1.3 PBMC Proliferation

All animals in the present studies responded to *in vitro* stimulation with ConA and LPS 055:B5 indicating peripheral T and B cells are capable of responding to an antigenic challenge. The use of two mitogens that preferentially stimulate either T cells (Barta and Barta, 1993) or B cells (Wechsler-Reya and Monroe, 1996) allowed the assessment of both T and B cell populations. *In vitro* proliferation of B cells was lower than T cells in both SSL (Chapter 2) and HS (Chapter 4) similar to previous observations in HS (Levin *et al.*, 2005). The lower proliferation of B cells is likely the result of a lower number of B cells than T cells in the peripheral blood as observed in other mammals (Byrne *et al.*, 2000; Faldyna *et al.*, 2001). A decrease in T cell proliferation but no change in B cell proliferation was associated with increasing age during the postnatal period in SSL (Chapter 2). The observed age related changes in cell-mediated immunity were not associated with a decrease in circulating lymphocyte or monocytes during the postnatal period in SSL (Chapter 2). The observed changes in T cell proliferation associated with age in SSL (Chapter 2) and lower proliferation of B cells compared to T cells in SSL pups (Chapter 2) and juvenile HS (Chapter 4) may be related to the ratio of T and B cells in circulation; however, I did not quantify the number of circulating T and B cells. The ratio of T and B cells has not been explored in pinnipeds and will need to be determined in future studies. These studies will depend on the cross reactivity of commercially available antibodies or development of species-specific antibodies.

In addition to establishing normal ranges for T and B cells in pinnipeds, the application of immunophenotying lymphocyte subpopulations would give further insight into leukocyte trafficking during an acute stress response. GC have been shown to negatively affect the immune system (Marketon and Glaser, 2008) with administration of ACTH or cortisol producing characteristic changes in circulating populations of

leukocytes often referred to the stress leukogram. As part of the stress leukogram, lymphocyte counts decrease in mammals (Dhabhar *et al.*, 1995; Dhabhar *et al.*, 1996). Juvenile HS displayed the classic stress leukogram 240 min after administration of exogenous ACTH (Chapter 4), while no change in lymphocyte counts was associated with the rookery disturbance and handling of SSL pups (Chapter 2). In rats the reduction in circulating lymphocytes associated with restraint stress was due to a decrease in both circulating B and T cells; however, the reduction of B cells was greater (Dhabhar *et al.*, 1995). Future studies to determine the number and proportion of circulating subpopulations of lymphocytes would provide insight into the relationship between changes observed in the number of circulating lymphocytes and the differences or changes in proliferation of T and B cells in response to age and acute stressors.

5.1.4 Effect of Handling and Sampling

In the present study, cortisol and all forms of thyroid hormones quantified decreased when regressed against elapsed time since arrival on rookery in wild SSL pups (Chapter 3). The decrease in total and free T_4 and total T_3 is likely the result of the initial rookery disturbance and associated increased activity of pups. However, the elapsed time is by necessity later in the day and therefore the decrease in thyroid hormones may not solely be related to the rookery disturbance but rather the result of increasing temperatures as the day progressed. Further, cortisol concentrations influenced total and differential leukocytes counts in-juvenile harbor seal (HS) (Chapter 4). These findings highlight the importance of assessing the impact of potential stressors such as rookery disturbance and animal handling when sampling pinnipeds.

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Appendix A. List of commercially available RIA used to quantify circulating hormone concentrations.

Hormone	Manufacturer	Catalog	Range	Sensitivity
Cortisol	Siemens	TK01	1-50 μg/dL	0.2 μg/dL
Aldosterone	Siemens	PITKAL-4	25-1200 pg/ml	11 pg/ml
Triiodothyronine (T_3)	Siemens	TKT31	20-600 ng/dL	7 ng/dL
Thyroxin (T_4)	Siemens	TKT41	0.5-24 μg/dL	0.25 μg/dL
Free thyroxin (T_4)	Siemens	PITKF4-5	0.1-10 ng/dL	0.01 ng/dL
Leptin	Linco	XL-85K	1-50 ng/ml	0.5 ng/ml

Vacutainer CPT with sodium citrate (8 ml, BD 362761; 4 ml, BD)
Cell Proliferation Biotrak ELISA system, version 2 (GE Healthcare Bio-Sciences Corp., RPN 250)
Concanavalin A (Sigma Aldrich, C 04152)
Lipopolysaccharide from *Escherichia coli* 055:B5 (Sigma Aldrich, L-2880)

Appendix B. Standard Operating Protocol for Aldosterone Assays

OBJECTIVE: To determine aldosterone concentration in unextracted sera in Steller sea lions and harbor seals.

HEALTH AND SAFETY:
Proper lab attire including lab coats, gloves and safety glasses are required as needed or as indicated by MSDSs for chemicals being tested.

PERSONNEL/TRAINING/RESPONSIBILITIES:
Any lab employee or student familiar with the equipment and laboratory techniques and trained in this and any referenced SOPs may perform this procedure.

REQUIRED AND RECOMMEMDED MATERIALS:

Aldosterone coat-a-count kit (Siemens cat #PITKAL-4)
VWR lab marker
(4) non coated tubes
Serum samples

PROCEDURE:

1. Allow sera to thaw and come to room temperature.
2. Label tubes.
3. Pipette 50 µl of zero calibrator A into NSB and A tubes.
4. Pipette 50 µl of remaining calibrator, controls.
5. Pipette serum into the appropriate tubes.
6. Add 0.250 ml ^{125}I Aldosterone to every tube
7. Gently mix
8. Incubate for 18 hrs at room temperature
9. Decant thoroughly, removing all visible moisture.
10. Count for 1 minute in the gamma counter.

Tubes

1. TC Plain tube
2. "
3. NSB Plain tube
4. "
5. A (0 pg/ml)
6. "
7. B (25 pg/ml)
8. "
9. C (50 pg/ml)
10. "
11. D (100 pg/ml)
12. "
13. E (200 pg/ml)
14. "
15. F (600 pg/ml)
16. "
17. G (1200 pg/ml)
18. "
19. Control Low
20. "
21. Control High
22. "
23. Sample #1
24"

Appendix C. Standard Operating Protocol for Cortisol Assays

OBJECTIVE: To determine cortisol concentrations in unextracted sera from Steller sea lions and harbor seals.

HEALTH AND SAFETY:
Proper lab attire including lab coats, gloves and safety glasses are required as needed or as indicated by MSDSs for chemicals being tested.

PERSONNEL/TRAINING/RESPONSIBILITIES:
Any lab employee or student familiar with the equipment and laboratory techniques and trained in this and any referenced SOPs may perform this procedure. Personnel must be trained for radioactive techniques.

REQUIRED AND RECOMMEMDED MATERIALS:

Cortisol Coat-a-count kit (Siemens TK01)
Serum samples
VWR lab marker
(4) non coated tubes

PROCEDURE:

1. Allow sera to thaw and come to room temperature.
2. Label tubes.
3. Pipette 12.5 µl of zero calibrator A into:
    a. NSB tubes
    b. A calibrator tube
4. Pipette 12.5 µl of remaining calibrator and samples into the appropriate tubes.
5. Add 500 µl of ^{125}I Cortisol to every tube.
6. Vortex
7. Incubate at 37°C for 45 min.
8. Decant supernatant (except TC 1, 2).
9. Count for 1 minute in a gamma counter.

Tubes

1. Total Count
2. "
3. NSB
4. "
5. 0 µg/dl (A)
6. "
7. 1 µg/dl (B)
8. "
9. 5 µg/dl (C)
10. "
11. 10 µg/dl (D)
12. "
13. 20 µg/dl (E)
14. "
15. 50 µg/dl (F)
16. "
17. Control 4
18. "
19. Control 5
20. "
21. Control 6
22. "
23. Sample #1
24. "

Appendix D. Standard Operating Protocol total thyroxine (T_4) Assays
OBJECTIVE: To determine total T_4 levels in sera from harbor seals and Steller sea lions.

HEALTH AND SAFETY:
Proper lab attire including lab coats, gloves and safety glasses are required as needed or as indicated by MSDSs for chemicals being tested.

PERSONNEL/TRAINING/RESPONSIBILITIES:
Any lab employee or student familiar with the equipment and laboratory techniques and trained in this and any referenced SOPs may perform this procedure. Personnel must be trained for RADIOACTIVE techniques.

REQUIRED AND RECOMMEMDED MATERIALS:

Total T_4 RIA kit (Siemens TKT41)
Serum samples
VWR marker
(4) non coated tubes

PROCEDURE:

1. Allow kit to come to room temperature.
2. Allow sera to thaw and come to room temperature.
3. Label tubes
    a. Plain tubes- (2) Total Count; (2) NSB
    b. Remaining tubes are coated tubes
4. Pipette 12.5 µ l of zero calibrator A into:
    a. NSB tubes
    b. A calibrator tube
5. Pipette 12.5 µ l of remaining calibrator and samples into the appropriate tubes.
6. Add 500 µ l of ^{125}I Total T_4 to every tube.
7. Vortex
8. Incubate at 37°C for 60 min.
9. Decant supernatant (except TC 1, 2) - remaining inverted for at least 2-3 min.
10. Count for 1 minute in a gamma counter.

Tubes
1. Total Count
2. "
3. NSB
4. "
5. 0 µg/dl (A)
6. "
7. 1 µ/dl (B)
8. "
9. 4 µg/dl (C)
10. "
11. 10 µg/dl (D)
12. "
13. 16 µg/dl (E)
14. "
15. 24 µg/dl (F)
16. "
17. Control low (B/C)
18. "
19. Control High (E/F)
20. "
21. Sample #1
22. "

Appendix E. Standard Operating Protocol for Free thyroxine (T_4) Assays

OBJECTIVE: To determine free T_4 levels in unextracted sera from harbor seals and Steller sea lions.

HEALTH AND SAFETY:
Proper lab attire including lab coats, gloves and safety glasses are required as needed or as indicated by MSDSs for chemicals being tested.

PERSONNEL/TRAINING/RESPONSIBILITIES:
Any lab employee or student familiar with the equipment and laboratory techniques and trained in this and any referenced SOPs may perform this procedure. Personnel must be trained for RADIOACTIVE techniques.

REQUIRED AND RECOMMEMDED MATERIALS:

Total T_4 RIA kit (Siemens PITKF4-5)
Sera samples
VWR marker
(4) non coated tubes

PROCEDURE:

1. Allow kit to come to room temperature.
2. Allow sera to thaw and come to room temperature.
3. Label tubes
    a. Plain tubes- (2) Total Count; (2) NSB
    b. Remaining tubes are coated tubes
4. Pipette 25 µl of zero calibrator A into:
    a. NSB tubes
    b. A calibrator tube
5. Pipette 25 µl of remaining calibrator and samples into the appropriate tubes.
6. Add 500 µl of ^{125}I free T_4 to every tube.
7. Vortex
8. Incubate at 37°C for 60 min.
9. Decant supernatant (except TC 1, 2)- remaining inverted for at least 2-3 min.
10. Count for 1 minute in a gamma counter.

Tubes

1. Total Count
2. "
3. NSB
4. "
5. 0 ng/dl (A)
6. "
7. 0.1 ng/dl (B)
8. "
9. 0.5 ng/dl (C)
10. "
11. 1.3 ng/dl (D)
12. "
13. 2.2 ng/dl (E)
14. "
15. 4.8 ng/dl (F)
16. "
17. 10 ng/dl (G)
18. "
19. Cntrl low (B/C)
20. "
21. Cntrl High (F/G)
22. "
23. Sample #1
24. "

Appendix F. Standard Operating Protocol for Total triiodothyronine (T_3) Assays

OBJECTIVE: To determine total T_3 concentrations in unextracted sera from harbor seals and Steller sea lions.

HEALTH AND SAFETY:
Proper lab attire including lab coats, gloves and safety glasses are required as needed or as indicated by MSDSs for chemicals being tested.

PERSONNEL/TRAINING/RESPONSIBILITIES:
Any lab employee or student familiar with the equipment and laboratory techniques and trained in this and any referenced SOP may perform this procedure. Personnel must be trained for RADIOACTIVE techniques.

REQUIRED AND RECOMMEMDED MATERIALS:

Total T_3 RIA kit (Siemens TKT31)
Serum samples
VWR marker

PROCEDURE:

1. Allow kit to come to room temperature.
2. Allow sera to thaw and come to room temperature.
3. Label tubes
    a. Plain tubes- (2) Total Count; (2) NSB
    b. Remaining tubes are coated tubes
4. Pipette 50 ul of zero calibrator A into:
    a. NSB tubes
    b. A calibrator tube
5. Pipette 50 ul of remaining calibrator and samples into the appropriate tubes.
6. Add 500 ul of ^{125}I total T3to every tube.
7. Vorex
8. Incubate at 37°C for 2 hours.
9. Decant supernatant (except TC 1,2)- remaining inverted for at least 2-3 min.
10. Count for 1 minute in a gamma counter.

Tubes	9. 20 ng/dl (B)	17. 600 ng/dl (F)
	10. "	18. "
1. Total Count	11. 50 ng/dl (C)	19. Control low (B/C)
2. "	12. "	20. "
3. NSB	13. 100 ng/dl (D)	21. Control high (E/F)
4. "	14. "	22. "
5. 0 ng/dl (A)	15. 200 ng/dl (E)	23. Sample #1
6. "	16. "	24. "

Appendix G. Standard Operating Protocol for Leptin Assays

OBJECTIVE: To determine Leptin levels in sera from harbor seals.

HEALTH AND SAFETY:
Proper lab attire including lab coats, gloves and safety glasses are required as needed or as indicated by MSDSs for chemicals being tested.

PERSONNEL/TRAINING/RESPONSIBILITIES:
Any lab employee or student familiar with the equipment and laboratory techniques and trained in this and any referenced SOPs may perform this procedure.

REQUIRED AND RECOMMEMDED MATERIALS:

Leptin RIA kit (Linco XL-85K)
Serum samples (1:1)
VWR marker
Glass tubes

PROCEDURE:

Day 1

1. Allow kit to come to room temperature.
2. Allow sera to thaw and come to room temperature.
3. Label tubes.
4. Pipette 150 µl of assay buffer:
    a. NSB tubes (tube 3,4)
    b. Bo (tube 5,6)
5. Pipette 50 µl:
    a. Standards (tubes
    b. Quality controls (tubes
    c. Samples
6. Pipette 50 µl Mulit species Leptin antibody to tubes 5 on.
7. Vortex, cover, and incubate overnight (20-24 hrs) at 4°C water bath

Day 3

8. Pipette 50 µl of ^{125}I-Human Leptin to all tubes.
9. Vortex, cover, and incubate overnight (20-24 hrs) at 4°C water bath.

Day 4

10. Add 500 µl cold (4°C) Precipitating Reagent to all tubes except total count (tubes 1,2)
11. Vortex and incubate 20 minutes at 4°C.
12. Centrifuge at 4°C, 3000 g, 30 min
13. Decant supernatant (except TC 1,2)
14. Count all tubes on gamma counter for 1 minute

Tubes

1. Total Count
2. "
3. NSB
4. "
5. 0 ng/ml (A)
6. "
7. 1 ng/ml (B)
8. "
9. 2 ng/ml (B)
10. "
11. 5 ng/ml (D)
12. "
13. 10 ng/ml (E)
14. "
15. 20 ng/ml (F)
16. "
17. 50 ng/ml (G)
18. "
19. Control Low
20. "
21. Control High
22. "
23. E110
24. "

Appendix H. Standard Operating Protocol for cell preparation tubes (CPT™)

OBJECTIVE: To isolate and freeze peripheral blood mononuclear cells (PBMC) for lymphocyte proliferation assay.

HEALTH AND SAFETY:
Proper lab attire including lab coats, gloves and safety glasses are required as needed or as indicated by MSDSs for chemicals being tested.

PERSONNEL/TRAINING/RESPONSIBILITIES:
Any lab employee or student familiar with the equipment and laboratory techniques and trained in this and any referenced SOPs may perform this procedure.

REQUIRED AND RECOMMEMDED MATERIALS:

2 (8ml) Vacutainer CPT with sodium citrate (BD 362761) per animal
PBS **w/out** Ca^{2+} /Mg^{2+} (Celgro, 21-040-CV)
Freezing Media (RPMI-20 + 10% DMSO)
Cryovials
Sterile transfer Pipettes
Gloves
70% ETOH
VWR Marker

PROCEDURE:

1. Thaw freezing media. Keep cold.
2. Collect blood into the CPT tube using standard techniques (invert tube several times to mix blood and anticoagulant).
3. After collection, tube can be kept upright at room temperature for up to two hours, before centrifugation.
4. Remix blood sample immediately prior to centrifugation (gently invert 8-10x)
5. Centrifuge tube for 20 min, 20°C, 1500 x g. Once centrifuged tube can be stored upright at 4°C for up to 24 hrs until processed further.
6. From this point on should be done under the sterile hood.
7. Using the sterile pipette carefully remove the top clear layer of liquid (only 1-2 ml) and discard.
8. Transfer the remaining contents i.e. the "fuzzy" layer above the gel and remaining clear solution into a 15 ml centrifuge tube containing 8mls of PBS.
9. Mix cells and PBS by gentle inversion-5x.
10. Centrifuge for 8 min at 950 rpm, 20°C.
11. Decant the supernatant without disturbing cell pellet.
12. Add 1 ml freezing media and mix thoroughly by repeated pippeting.
13. Transfer cells and freezing media into cryovials.
14. Place cryovials in freezing chamber and store at -80°C until the next day.
15. Remove cryovials from freezing chamber and place in liquid N_2 dewar.

Appendix I. Standard Operating Protocol for Sodium Heparin Tubes

OBJECTIVE: To freeze WBC for future lymphocyte isolation and proliferation assay.

10 ml Na- Heparin green top tube (GT)
Freezing Media
Cryovials
Transfer Pipettes
Gloves
70% ETOH
VWR Marker

PROCEDURE:

1. Thaw freezing media. Keep cold.
2. Collect blood into the GT tube using standard techniques (invert tubes 4-5x to mix blood and anticoagulant).
3. After collection, tube can be upright and chilled until centrifugation.
4. Remix blood sample immediately prior to centrifugation (gently invert 4-5x).
5. Centrifuge tube for 10 min at 950 rpm.
6. From this point on should be done under the sterile hood.
7. Using the individually wrapped sterile pipette carefully remove the top clear layer of plasma without disturbing the white buffy layer.
8. Transfer plasma into cryovials and store at -80°C.
9. Gently transfer the buffy coat (i.e. "fuzzy" layer above RBC) into 1.2 ml cryovials.
10. Using a new individually wrapped sterile pipette add 1 ml freezing media to the WBC cryovial and mix thoroughly by gently inverting the cryovial containing the WBC and Freezing media.
11. Place cryovials in freezing chamber and store at -80°C until the next day.
12. Remove cryovials from freezing chamber and place in liquid N_2 dewar.

Appendix J: Standard Operating Protocol for PBMC Proliferation Assay

I. Thawing Cells

1. Remove vial from liquid N_2 and thaw quickly in 37°C water bath.
2. Transfer contents of vial into 10 mls of RPMI-10 Media.
3. Centrifuge for 10 min, 1000 rpm, 20°C (Wash 1).
4. Aspirate supernatant without disturbing cell pellet.
5. Re-suspend cells; add 10 ml RPMI-10.
6. Allow cells to sit at room temperature for 2 hours.
7. Centrifuge for 10 min, 1000 rpm, 20°C (Wash 2).
8. Re-suspend cells; add 5 ml RPMI-10
9. Transfer 50 µl of cells to eppendorf tube with 200 µl RPMI-10,
10. Count cells.
11. Bring volume up to 10 ml Centrifuge for 10 min, 1000 rpm, 20°C (Wash 3).
12. Re-suspend cells in C-RPMI-10 at a final concentration of 4 $x10^6$ cells/ml; ($2x10^5$cells/50 µl)

II. Preparing Mitogens

Reagents Needed:
C- RPMI-10
Stock mitogen(s) 1 mg/ml
Concanavalin A (Sigma, cat # C 04152)
LPS 055:B5 (Sigma, cat # L-2880)

13. Based on the experiment, determine which concentration of mitogen requires the highest volume.
14. Using this volume as the minimum, add an additional amount.
15. Continue with this process until the lowest concentration needed is made.

III. Plating Cells

Place the following in the hood:
Eight channel pipette
Pipette tips
Sterile boat (one for each animal)

16. Label the plate (s).
17. With the eight channel pipette, aliquot the mitogens (50 µl/well) into their respective wells according to the schematic.
18. Once all of the mitogens have been plated, precede to aliquot the cells into their respective wells using 50 µl/well.
19. Cover plates when finished and observe cells under the microscope.
20. Place in 37°C, 5% CO_2 incubator for 48 hrs.

Make sure enough CO_2 is left in the tanks and that the metal tray at the bottom of the incubator contains enough sterile water.

IV. Labeling the Cells with BrdU

Allow all reagents to reach room temperature before assay.
Mix samples and all reagents prior to use.
Avoid excessive foaming of reagents.

21. Dilute the BrdU labeling reagent (bottle 1) 1:100 with C-RPMI-10 [100μM].
22. Add 10 μl/well BrdU labeling solution.
23. Re-incubate cell cultures for an additional 18 hrs at 37°C, 5% CO_2.
24. Centrifuge the plate at 1500 rpm for 10 min, 20°C.
25. Remove media by tapping.
26. Dry cells in drying oven (60°C) for 1 hr.
27. Add 200μl/well fixative and incubate for 30 min at room temperature.
28. Remove fixative by tapping.
29. Add 200 μl/well blocking buffer.
30. Remove blocking buffer by tapping.
31. Add 100 μl/well peroxidase-labelled anti-BrdU working solution (**1:100** antibody dilution solution)
32. Incubate for 90 min at room temperature.
33. Remove antibody conjugate by tapping.
34. Rinse wells three times with 200 μl/well washing solution.
35. Immediately dispense 100 μl of room temperature equilibrated TMB substrate into all wells.
36. Cover the plate and mix at room temperature until color development is sufficient (5-30 min).
37. Add 25 μl of 1M sulphuric acid (H_2SO_4) to each well in order to stop reaction.
38. Determine the optical density in the plate reader at 450 nm within 5 min.

Cell Count:

1. Label 0.5 mL sterile eppendorf tubes for each animal.
2. Using a sterile 1ml pipette add 200 μL C-RPMI-5 into each eppendorf tube.
3. Using a sterile eppendorf tip add 50 μL cells into the appropriate tube.
4. Mix well.
5. The counts are done non-sterilely so remove just the eppendorf tubes from the hood.
6. In a 96 well flat-bottom plate mix 50 μL of the cells/media with 50 μL Trypan blue.
7. Gives a total dilution of 1:10.
8. Using a hemacytometer and microscope count four grid squares for each animal.

Calculations:
Total cells/4= (Ave cell) (10) (10^4) = # cells/ml

Appendix K. Solutions

1M sulphuric acid (H_2SO_4)

560 µl concentrated sulphuric acid to 8 ml of **ice-cold** distilled water.
Carefully mix and make up to 10 ml with water

All steps for the following solutions should be performed using sterile techniques in a sterile hood. Wash hood with 70% alcohol before and after use.

C-RPMI-10 Media

RPMI 1640 w/out L-glutamine (Gibco, 21870-092)
50 ml FBS (Hyclone, SH3007103HI)
5 ml Glutamate-Penicillin-Streptomycin (Gibco, 10378-016)
5 ml 100 mM Sodium Pyruvate (Gibco, 11360-070)
5 ml 100x Nonessential Amino Acids (Gibco, 11140-050)
5 ml 1M HEPES (Gibco, 15630-080)
500 µl 0.05 M 2-mercaptoethanol (Sigma, M7522-100ML)
Store at 4°C

0.05 M 2-mercaptoethanol

20 µl 14.3 M 2-mercaptoethanol (Sigma, # M-7522)
5.6 ml RPMI-10

RPMI-10 Media

RPMI 1640 w/out L-glutamine (Gibco, 21870-092)
50 ml FBS (Hyclone, SH3007103HI)
5 ml Glutamate-Penicillin-Streptomycin (Gibco, 10378-016)
Store at 4°C

LPS 055:B5 stock [1mg/ml]

For every mg of LPS 055:B55 add 1 ml of C-RPMI-10. Mix thoroughly and aliquot into sterile 1 µl eppendorf and store at –80°C.

ConA stock [1mg/ml]

For every mg of Con A add 1 ml of C-RPMI-10. Mix thoroughly and aliquot into sterile 500 µl eppendorf and store at –80°C.

Freezing Media

RPMI-10 Media
20% FBS (Hyclone, SH3007103HI)
10% DMSO (Sigma)
Mix thoroughly and aliquot 5-10 ml aliquots into sterile 15 ml conical tubes and store at –80°C.